



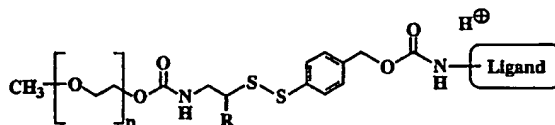
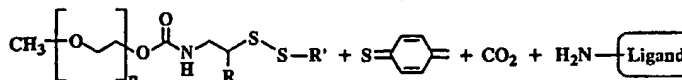
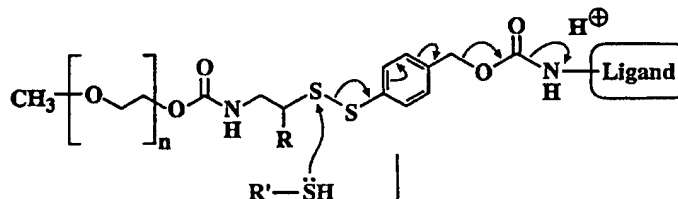
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US00/10830 (22) International Filing Date: 21 April 2000 (21.04.00) (30) Priority Data: 60/130,897 23 April 1999 (23.04.99) US (71) Applicant: ALZA CORPORATION [US/US]; 1900 Charleston Road, P.O. Box 7210, Mountain View, CA 94039-7210 (US). (72) Inventor: ZALIPSKY, Samuel; 1202 Truman Street, Redwood City, CA 94061 (US). (74) Agents: SIMBOLI, Paul, B. et al.; ALZA Corporation, 1900 Charleston Road, P.O. Box 7210, Mountain View, CA 94039-7210 (US).	(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>	

(54) Title: RELEASABLE LINKAGE AND COMPOSITIONS CONTAINING SAME

(57) Abstract

A compound comprised of a hydrophilic polymer covalently yet reversibly linked to an amine-containing ligand through a dithiobenzyl linkage is described.

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RELEASABLE LINKAGE AND COMPOSITIONS CONTAINING SAME**Field of the Invention**

The present invention relates to a compound comprised of a hydrophilic polymer,
5 such as polyethyleneglycol, cleavably linked to an amine-containing ligand, which in
preferred embodiments can be an amine-containing lipid, drug or protein. The
compounds are cleavable under mild thiolytic conditions to regenerate the amine-
containing ligand in its original form.

10

Background of the Invention

Hydrophilic polymers, such as polyethylene glycol (PEG), have been used for
modification of various substrates, such as polypeptides, drugs and liposomes, in order to
reduce immunogenicity of the substrate and/or to improve its blood circulation lifetime.

For example, parenterally administered proteins can be immunogenic and may
15 have a short pharmacological half-life. Proteins can also be relatively water insoluble.
Consequently, it can be difficult to achieve therapeutically useful blood levels of the
proteins in patients. Conjugation of PEG to proteins has been described as an approach to
overcoming these difficulties. Davis *et al.* in U.S. Pat. No. 4,179,337 disclose
conjugating PEG to proteins such as enzymes and insulin to form PEG-protein
20 conjugates having less immunogenicity yet which retain a substantial proportion of
physiological activity. Veronese *et al.* (*Applied Biochem. and Biotech.*, 11:141-152
(1985)) disclose activating polyethylene glycols with phenyl chloroformates to modify a
ribonuclease and a superoxide dimutase. Katre *et al.* in U.S. Pat. Nos. 4,766,106 and
4,917,888 disclose solubilizing proteins by polymer conjugation. PEG and other
25 polymers are conjugated to recombinant proteins to reduce immunogenicity and increase
half-life. (Nitecki *et al.*, U.S. Pat. No. 4,902,502; Enzon, Inc., PCT/US90/02133).
Garman (U.S. Patent No. 4,935,465) describes proteins modified with a water soluble
polymer joined to the protein through a reversible linking group.

However, PEG-protein conjugates described to date suffer from several
30 disadvantages. For example, modification of the protein with PEG often inactivates the
protein so that the resulting conjugate has poor biological activity. Typically in the

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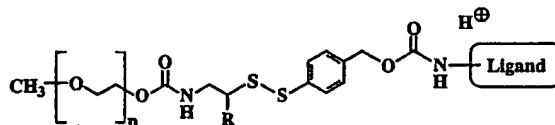
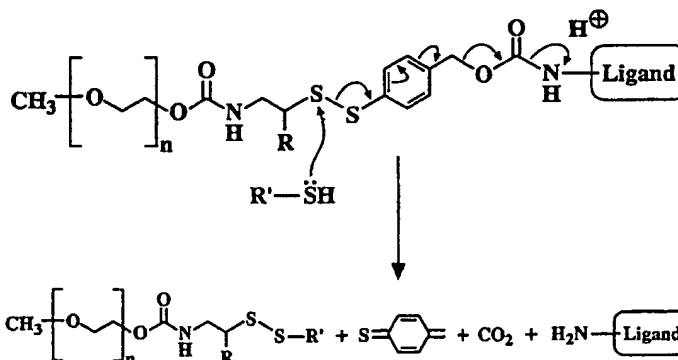
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RELEASABLE LINKAGE AND COMPOSITIONS CONTAINING SAME

Field of the Invention

The present invention relates to a compound comprised of a hydrophilic polymer,
5 such as polyethyleneglycol, cleavably linked to an amine-containing ligand, which in preferred embodiments can be an amine-containing lipid, drug or protein. The compounds are cleavable under mild thiolytic conditions to regenerate the amine-containing ligand in its original form.

Background of the Invention

Hydrophilic polymers, such as polyethylene glycol (PEG), have been used for modification of various substrates, such as polypeptides, drugs and liposomes, in order to reduce immunogenicity of the substrate and/or to improve its blood circulation lifetime.

For example, parenterally administered proteins can be immunogenic and may
15 have a short pharmacological half-life. Proteins can also be relatively water insoluble. Consequently, it can be difficult to achieve therapeutically useful blood levels of the proteins in patients. Conjugation of PEG to proteins has been described as an approach to overcoming these difficulties. Davis *et al.* in U.S. Pat. No. 4,179,337 disclose conjugating PEG to proteins such as enzymes and insulin to form PEG-protein
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25 polymers are conjugated to recombinant proteins to reduce immunogenicity and increase half-life. (Nitecki *et al.*, U.S. Pat. No. 4,902,502; Enzon, Inc., PCT/US90/02133). Garman (U.S. Patent No. 4,935,465) describes proteins modified with a water soluble polymer joined to the protein through a reversible linking group.

However, PEG-protein conjugates described to date suffer from several
30 disadvantages. For example, modification of the protein with PEG often inactivates the protein so that the resulting conjugate has poor biological activity. Typically in the

prior art to date, it is desired to have the PEG stably linked to the protein so that the beneficial properties provided by PEG remain. Another problem with some protein PEG conjugates is that upon decomposition of the conjugate undesirable products may be formed.

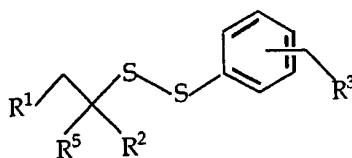
5 PEG has also been described for use in improving the blood circulation lifetime of liposomes (U.S. Patent No. 5,103,556). Here, the PEG is covalently attached to the polar head group of a lipid in order to mask or shield the liposomes from being recognized and removed by the reticuloendothelial system. Liposomes having releasable PEG chains have also been described, where the PEG chain is released from
10 the liposome upon exposure to a suitable stimulus, such as a change in pH (PCT/US97/18813). However, release of the PEG chain from the liposome suffers from the drawback that the decomposition products are chemically modified and can have unpredictable, potentially negative effects *in vivo*.

Summary of the Invention

15 Accordingly, it is an object of the invention to provide a compound where a ligand is covalently yet reversibly linked to a hydrophilic polymer. Upon cleavage of the linkage, the ligand in its native form is regenerated.

In one aspect, the invention includes a compound having the general structure:

20



wherein R¹ is a hydrophilic polymer comprising a linkage for attachment to the dithiobenzyl moiety; R² is selected from the group consisting of H, alkyl and aryl; R³ is
25 selected from the group consisting of O(C=O)R⁴, S(C=O)R⁴, and O(C=S)R⁴; R⁴ comprises an amine-containing ligand; and R⁵ is selected from the group consisting of H, alkyl and aryl; and where orientation of CH₂-R³ is selected from the ortho position and the para position.

In one embodiment, R⁵ is H and R² is selected from the group consisting of CH₃,

C_2H_5 and C_3H_8 . In another embodiment, R^2 and R^5 are alkyls.

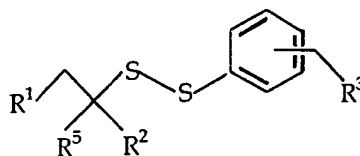
In another embodiment, the amine-containing ligand R^4 is selected from the group consisting of a polypeptide, an amine-containing drug and an amine-containing lipid. In an embodiment where the amine-containing ligand R^4 is an amine-containing lipid, the lipid includes either a single hydrocarbon tail or a double hydrocarbon tail. In one preferred embodiment, the lipid is a phospholipid having a double hydrocarbon tail.

The hydrophilic polymer R^1 can be, in yet another embodiment, selected from the group consisting of polyvinylpyrrolidone, polyvinylmethylether, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyloxazoline, polyhydroxypropyl-methacrylamide, polymethacrylamide, polydimethyl-acrylamide, polyhydroxypropylmethacrylate, polyhydroxyethylacrylate, hydroxymethylcellulose, hydroxyethylcellulose, polyethyleneglycol, polyaspartamide, copolymers thereof, and polyethyleneoxide-polypropylene oxide.

In one preferred embodiment, the hydrophilic polymer R^1 is polyethyleneglycol. In another embodiment, when R^1 is polyethylene glycol, R^5 is H and R^2 is CH_3 or C_2H_5 .

In still another embodiment, the amine-containing ligand R^4 is a polypeptide. The polypeptide can be, in another embodiment, a recombinant polypeptide. Exemplary and preferred polypeptides include cytokines, such as interferons, interleukins, and growth factors, and enzymes.

In another aspect, the invention includes a composition comprising a conjugate obtainable by reaction with a compound having the general structural formula:



wherein R^1 is a hydrophilic polymer comprising a linkage for attachment to the dithiobenzyl moiety; R^2 is selected from the group consisting of H, alkyl and aryl; R^3 is selected from the group consisting of $O(C=O)R^4$, $S(C=O)R^4$, and $O(C=S)R^4$; R^4 comprises a leaving group; and R^5 is selected from the group consisting of H, alkyl and aryl; and where orientation of CH_2-R^3 is selected from the ortho position and the para

position. The composition also includes a pharmaceutically-acceptable carrier, such as saline, buffer or the like.

In one embodiment of this aspect, R^2 is selected from the group consisting of CH_3 , C_2H_5 and C_3H_7 .

5 In another embodiment, R^3 is $O(C=O)R^4$ and R^4 is a hydroxy- or oxy-containing leaving group. The leaving group, in another embodiment, is derived from a compound selected from the group consisting of chloride, *para*-nitrophenol, *ortho*-nitrophenol, N-hydroxy-tetrahydrophthalimide, N-hydroxysuccinimide, N-hydroxy-glutarimide, N-hydroxynorbornene-2,3-dicarboxyimide, 1-hydroxybenzotriazole, 3-hydroxypyridine, 4-
10 hydroxypyridine, 2-hydroxypyridine, 1-hydroxy-6-trifluoromethylbenzotriazole, imidazole, triazole, N-methyl-imidazole, pentafluorophenol, trifluorophenol and trichlorophenol.

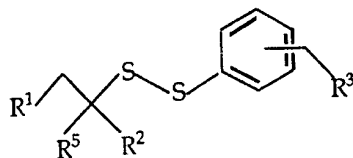
In one embodiment, the claimed compound is reacted with an amine-containing
15 ligand that displaces R^4 to form a conjugate that includes the amine-containing ligand. For example, the amine-containing ligand can be a phospholipid.

In a preferred embodiment, the hydrophilic polymer R^1 is polyethyleneglycol, R^5 is H and R^2 is CH_3 or C_2H_5 .

In yet another aspect of this embodiment, the composition containing the
20 conjugate comprises a liposome. The liposome can further comprise an entrapped therapeutic agent.

In another embodiment, the amine-containing ligand comprises a polypeptide.

In yet another aspect, the invention includes a liposome composition comprising
liposomes which include a surface coating of hydrophilic polymer chains wherein at
25 least a portion of the hydrophilic polymer chains have the general structure:

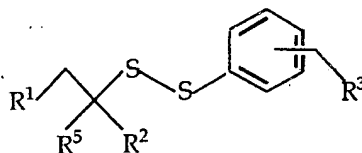


wherein R^1 is a hydrophilic polymer comprising a linkage for attachment to the dithiobenzyl moiety; R^2 is selected from the group consisting of H, alkyl and aryl; R^3 is

selected from the group consisting of $O(C=O)R^4$, $S(C=O)R^4$, and $O(C=S)R^4$; R^4 comprises an amine-containing ligand; and R^5 is selected from the group consisting of H, alkyl and aryl; and where orientation of CH_2-R^3 is selected from the ortho position and the para position. The liposomes have a longer blood circulation lifetime than
 5 liposomes having hydrophilic polymer chains joined to the liposome via an aliphatic disulfide linkage.

In one embodiment, the liposome further comprises an entrapped therapeutic agent.

In still another aspect, the invention includes a method for improving the blood
 10 circulation lifetime of liposomes having a surface coating of releasable hydrophilic polymer chains. The method includes preparing liposomes that have between about 1 % to about 20 % of a compound having the general structure:



15 wherein R^1 , R^2 , R^3 , and R^5 are as described above and R^4 comprises an amine-containing lipid.

In a preferred embodiment of this aspect, R^5 is H and R^2 is selected from the group consisting of CH_3 , C_2H_5 and C_3H_7 .

In another embodiment, the amine-containing lipid comprises a phospholipid and
 20 R^1 is polyethyleneglycol.

In this aspect, the liposomes can further comprise an entrapped therapeutic agent.

These and other objects and features of the invention will be more fully appreciated when the following detailed description of the invention is read in conjunction with the accompanying drawings.

25

Brief Description of the Drawings

Fig. 1A shows an embodiment of the invention where the dithiobenzyl (DTB) links a methoxy-polyethylene glycol (mPEG) moiety and the amine-containing ligand;

Fig. 1B shows the products after thiolytic cleavage of the compound in Fig. 1A;

Fig. 2 illustrates a synthetic reaction scheme for synthesis of the mPEG-DTB-amine-lipid, where the amine-ligand is the lipid distearoylphosphatidylethanolamine (DSPE);

Fig. 3 illustrates the thiolytic cleavage mechanism of a *para*-dithiobenzyl urethane (DTB)-linked mPEG-DSPE conjugate;

Figs. 4A-4B show a synthetic reaction scheme for preparation of an mPEG-DTB-DSPE compound in accord with the invention where the DTB linkage is sterically hindered by an alkyl group;

Fig. 5 shows another synthetic reaction scheme for preparation of an mPEG-DTB-ligand compound in accord with the invention;

Fig. 6A is a synthetic reaction scheme for synthesis of an mPEG-DTB-lipid which upon thiolytic cleavage yields a cationic lipid;

Fig. 6B shows the products after thiolytic cleavage of the compound in Fig. 6A;

Fig. 7A shows the rate of cleavage of *ortho*-mPEG-DTB-DSPE and *para*-mPEG-DTB-DSPE conjugates in solution to form micelles in buffer alone (*ortho*-conjugate (*); *para*-conjugate (+)) and in the presence of 150 μ M cysteine (*ortho*-conjugate (open circles); *para*-conjugate (open squares));

Fig. 7B shows the rate of cleavage of micellar mPEG-DTB-DSPE conjugates as described in Fig. 7A and of *ortho*-mPEG-DTB-DSPE (solid circles) and *para*-mPEG-DTB-DSPE (solid squares) conjugates formulated in liposomes and incubated in the presence of 150 μ M cysteine;

Figs. 8A-8B show percentage of content release of entrapped fluorophore from liposomes comprised of DOPE:*ortho*-mPEG-DTB-DSPE (Fig. 8A) or of DOPE:*para*-mPEG-DTB-DSPE (Fig. 8B) incubated in the presence of cysteine at the indicated concentrations;

Fig. 9A shows normalized percent release of entrapped fluorophore as a function of time for liposomes comprised of DOPE and *para*-mPEG-DTB-DSPE. The percent release of entrapped fluorophore is normalized with respect to percent release of fluorophore from liposomes incubated in the absence of cysteine. The release rate from liposomes incubated in the presence of cysteine at concentrations of 15 μ M (solid squares), 75 μ M (open

triangles), 150 μ M (X symbols), 300 μ M (open circles), 1500 μ M (solid circles), 3000 μ M (+ symbols), and 15000 μ M (open diamonds) is shown;

Fig. 9B shows normalized percent release of entrapped fluorophore as a function of time for liposomes comprised of DOPE and *para*-mPEG-MeDTB-DSPE. The percent
5 release of entrapped fluorophore is normalized with respect to percent release of fluorophore from liposomes incubated in the absence of cysteine. The release rate for liposomes incubated in the presence of cysteine at concentrations of 15 μ M (solid squares), 75 μ M (open triangles), 150 μ M (X symbols), 300 μ M (open circles), 1500 μ M (solid circles), 3000 μ M (+ symbols), and 15000 μ M (open diamonds) is shown;

10 Fig. 9C shows normalized percent release of entrapped fluorophore as a function of time for liposomes comprised of DOPE and mPEG-meDTB-distearoyl-glycerol compound of Fig. 6A. The percent release of entrapped fluorophore is normalized with respect to percent release of fluorophore from liposomes incubated in the absence of cysteine. The release rate of dye upon cleavage of the compound from liposomes incubated in the
15 presence of cysteine at concentrations of 15 μ M (solid squares), 75 μ M (open triangles), 150 μ M (X symbols), 300 μ M (open circles), 1500 μ M (solid circles), 3,000 μ M (+ symbols), and 15,000 μ M (open diamonds) is shown;

Fig. 10 is a plot showing the amount of liposomes, in counts per minute/mL of liposomes containing entrapped In^{111} , in blood samples taken from mice at various times
20 after injection of liposomes comprised of PHPC:cholesterol:mPEG-DTB-DSPE (55:40:5 molar ratio). One group of animals received a 200 μ L injection of 200 mM cysteine at time zero (solid squares). The control group was injection with saline at time zero (open circles);

Fig. 11A shows a synthetic reaction scheme for synthesis of an mPEG-DTB-
25 protein compound in accord with another embodiment of the invention;

Fig. 11B shows the decomposition products after thiolytic cleavage of the compound in Fig. 11A;

Fig. 12 is a rendering of a photograph of an sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile of lysozyme reacted for 15
30 minutes (Lane 1) or for 1 hour (Lane 2) with mPEG-MeDTB-nitrophenylchloroformate to form a mPEG-MeDTB-lysozyme conjugate, native

lysozyme (Lane 3), lysozyme reacted for 1 hour with mPEG-nitrophenylchloroformate (Lane 4), molecular weight markers (Lane 5), and the samples of Lanes 1-4 treated with 2% β -mercaptoethanol for 10 minutes at 70°C (Lanes 6-9);

Fig. 13 shows the decomposition products after thiolytic cleavage of the an mPEG-DTB-*p*-nitroanilide conjugate;

Fig. 14A shows the absorbance as a function of wavelength, in nm, of mPEG-MeDTB-*para*-nitroanilide (closed diamonds) and after *in vitro* incubation with 5 mM cysteine for 2 minutes (closed squares), 5 minutes (x symbols), 10 minutes (open squares), 20 minutes (triangles), 40 minutes (open diamonds) and 80 minutes (closed circles); and

Fig. 14B shows the amount of *para*-nitroanilide, in mole/L, released *in vitro* as a function of time, in minutes, from mPEG-MeDTB-*para*-nitroanilide conjugate incubated in the presence of 5 mM cysteine (closed circles), 1 mM cysteine (closed squares) and 0.15 mM cysteine (closed diamonds).

Detailed Description of the Invention

I. Definitions

"Polypeptide" as used herein refers to a polymer of amino acids and does not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, protein, and enzyme are included within the definition of polypeptide. This term also includes post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations, and the like.

"Amine-containing" intends any compound having a moiety derived from ammonia by replacing one or two of the hydrogen atoms by alkyl or aryl groups to yield general structures RNH_2 (primary amines) and R_2NH (secondary amines), where R is any hydrocarbyl group.

"Hydrophilic polymer" as used herein refers to a polymer having moieties soluble in water, which lend to the polymer some degree of water solubility at room temperature. Exemplary hydrophilic polymers include polyvinylpyrrolidone, polyvinylmethylether, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyloxazoline, polyhydroxypropyl-methacrylamide, polymethacrylamide,

polydimethyl-acrylamide, polyhydroxypropylmethacrylate, polyhydroxyethylacrylate, hydroxymethylcellulose, hydroxyethylcellulose, polyethyleneglycol, polyaspartamide, copolymers of the above-recited polymers, and polyethyleneoxide-polypropylene oxide copolymers. Properties and reactions with many of these polymers are described in U.S. Patent Nos. 5,395,619 and 5,631,018.

"Polymer comprising a reactive functional group" or "polymer comprising a linkage for attachment" refers to a polymer that has been modified, typically but not necessarily, at a terminal end moiety for reaction with another compound to form a covalent linkage. Reaction schemes to functionalize a polymer to have such a reactive functional group of moiety are readily determined by those of skill in the art and/or have been described, for example in U.S. Patent No. 5,613,018 or by Zalipsky *et al.*, in for example, *Eur. Polymer. J.*, 19(12):1177-1183 (1983); *Bioconj. Chem.*, 4(4):296-299 (1993).

"Recombinant" as in "recombinant polypeptide" implies joining of amino acids through laboratory manipulation into a desired sequence.

"Alkyl" as used herein intends a group derived from an alkane by removal of a hydrogen atom from any carbon atom: " C_nH_{2n+1} ". The groups derived by removal of a hydrogen atom from a terminal carbon atom of unbranched alkanes form a subclass of normal alkyl (n-alkyl) groups: $H[CH_2]_n$. The groups RCH_2- , R_2CH- (R not equal to H), and R_3C- (R not equal to H) are primary, secondary and tertiary alkyl groups respectively.

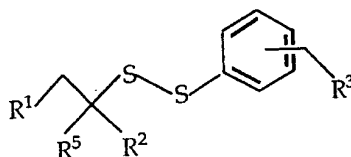
"Aryl" refers to a substituted or unsubstituted monovalent aromatic radical having a single ring (e.g., benzene) or two condensed rings (e.g., naphthyl). This term includes heteroaryl groups, which are aromatic ring groups having one or more nitrogen, oxygen, or sulfur atoms in the ring, such as furyl, pyrrole, pyridyl, and indole. By "substituted" is meant that one or more ring hydrogens in the aryl group is replaced with a halide such as fluorine, chlorine, or bromine; with a lower alkyl group containing one or two carbon atoms; nitro, amino, methylamino, dimethylamino, methoxy, halomethoxy, halomethyl, or haloethyl.

An "aliphatic disulfide" linkage intends a linkage of the form $R'-S-S-R''$, where R' and R'' are linear or branched alkyl chains that may be further substituted.

The following abbreviations are used herein: PEG, poly(ethylene glycol); mPEG, methoxy-PEG; DTB, dithiobenzyl; MeDTB, methyl-dithiobenzyl, EtDTB, ethyl-dithiobenzyl; DSPE, distearoyl phosphatidylethanolamine; DOPE, dioleoyl phosphatidylethanolamine; PHPC, partially hydrogenated phosphatidylcholine; MALDI-
 5 TOFMS, matrix-assisted laser desorption / ionization time-of-flight mass spectrometry.

II. The Compound of the Invention

In one aspect, the invention comprises a compound of the form:



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wherein R¹ comprises a hydrophilic polymer including functional group suitable for covalently attaching the polymer to the dithiobenzyl moiety. R² and R⁵ are independently selected to be H, an alkyl or an aryl, and, as will be seen, can be varied to tailor the rate of disulfide cleavage. For example, to achieve a faster rate of
 15 cleavage, R² and R⁵ are hydrogen. A slower rate of cleavage is achieved by sterically hindering the disulfide by selecting an alkyl or aryl for one or both of R² and R⁵. R³ comprises a linking moiety joined to R⁴, which comprises an amine-containing ligand. The linking moiety in preferred embodiments is O(C=O), S(C=O) or O(C=O). The amine-containing ligand R⁴ can be a primary or a secondary amine and can be selected
 20 from any number of substrates, including, but not limited to lipids, drugs, polypeptides, viruses, surfaces of biomaterials and aminoglycosides. In preferred embodiments, R⁴ is a primary or secondary amine-containing lipid, drug or polypeptide. In the compound of the invention, the orientation of the group CH₂-R³ can be either ortho or para.

Fig. 1A shows the structure of an exemplary compound in accord with the
 25 invention, where R¹ is the hydrophilic polymer methoxy-polyethylene glycol, mPEG=CH₃O(CH₂CH₂O)_n where *n* is from about 10 to about 2300, which corresponds to molecular weights of about 440 Daltons to about 100,000 Daltons. The molecular weight of the polymer depends to some extent on the selection of R³. In embodiments where R³ is an amine-containing lipid for use in a liposome a preferred range of PEG molecular weight

is from about 750 to about 10,000 Daltons, more preferably from about 2,000 to about 5,000 Daltons. The mPEG in this embodiment includes a urethane linking moiety. In embodiments where R^3 is an amine-containing polypeptide a preferred range of PEG molecular weight is from about 2,000 to about 40,000 Daltons, more preferably from about 2,000 to about 20,000 Daltons. It will be appreciated that R^1 can be selected from a variety of hydrophilic polymers, and exemplar polymers are recited above. It will also be appreciated that for some ligands, such as polypeptides, the molecular weight of the polymer may depend on the number of polymer chains attached to the ligand, where a larger molecular weight polymer is often selected when the number of attached polymer chains is small.

With continuing reference to Fig. 1a, R^2 and R^5 in this exemplary compound are H, however either or both R^2 and R^5 can also be a straight chain or branched alkyl or an aryl group. In a preferred embodiment, R^5 is H and R^2 is an alkyl, and several examples are given below. In the compound shown in Fig. 1A, R^3 takes the general form of $O(C=O)-(NH_2\text{-ligand})$, where the NH_2 -ligand can be any amine-containing polypeptide, drug or lipid, and specific examples of each embodiment are given below. R^3 can also be of the form $O(C=S)-(NH_2\text{-ligand})$ or $S(C=O)-(NH_2\text{-ligand})$.

Fig. 1B shows the mechanism of thiolytic cleavage of the mPEG-DTB- $(NH_2\text{-ligand})$ compound of Fig. 1A. The *ortho*- or *para*-dithiobenzyl carbamate moiety is cleavable under mild thiolytic conditions, such as in the presence of cysteine or other naturally-occurring reducing agents. Upon cleavage, the amine-containing ligand is regenerated in its natural, unmodified form. Studies in support of the invention, described below, show that natural, physiologic conditions *in vivo* are sufficient to initiate and achieve cleavage of the DTB linkage. It will be appreciated that a reducing agent can also be administered to artificially induce thiolytic conditions sufficient for cleavage and decomposition of the compound.

As noted above, R^3 takes the general form of a linking moiety, such as $O(C=O)$, $S(C=O)$ or $O(C=S)$ joined to an amine-containing ligand. In preferred embodiment, the amine-containing ligand comprises an amine-containing polypeptide, drug or lipid. Examples of these embodiments will now be described.

A. Amine-Containing Lipid

In one embodiment, the amine-containing ligand is an amine-containing lipid. Lipids as referred to herein intend water-insoluble molecules having at least one acyl chain containing at least about eight carbon atoms, more preferably an acyl chain containing between about 8-24 carbon atoms. A preferred lipid is a lipid having an amine-containing polar head group and an acyl chain. Exemplary lipids are phospholipids having a single acyl chain, such as stearylamine, or two acyl chains. Preferred phospholipids with an amine-containing head group include phosphatidylethanolamine and phosphatidylserine. The lipid tail(s) can have between about 12 to about 24 carbon atoms and can be fully saturated or unsaturated. One preferred lipid is distearylphosphatidylethanolamine (DSPE), however those of skill in the art will appreciate the wide variety of lipids that fall within this description. It will also be appreciated that the lipid can naturally include an amine group or can be derivatized to include an amine group. Other lipid moieties that do not have an acyl tail, such as cholesterolamine, are also suitable.

The synthesis of a polymer-DTB-lipid compound is schematically depicted in Fig. 2. mPEG derivatives (MW 2000 and 5000 Daltons) having a methoxycarbonyldithioalkyl end group were prepared by reacting 2-(methoxycarbonyldithio)ethaneamine with mPEG-chloroformate, which was readily prepared by phosgenation of dried mPEG-OH solution (Zalipsky, S., *et al.*, *Biotechnol. Appl. Biochem.* 15:100-114 (1992)). The former compound was obtained through 2-aminoethanethiol hydrochloride reaction with an equivalent amount of methoxycarbonylsulfonyl chloride, according to published procedures (Brois, S.J., *et al.*, *J. Amer. Chem. Soc.* 92:7629-7631 (1970); Koneko, T., *et al.*, *Bioconjugate Chem.* 2:133-141 (1991)). Both the *para* and *ortho* isomers of mercaptobenzyl alcohol (Grice, R., *et al.*, *J. Chem. Soc.* 1947-1954 (1963)) coupled cleanly with the resulting PEG-linked acyldisulfide, yielding mPEG bearing a dithio benzyl alcohol end group. Active carbonate introduction proceeded as with underivatized mPEG-OH, to give the *para*-nitrophenyl carbonate. Addition of DSPE in ethanolamine formed the desired mPEG-DTB-DSPE product. Both *ortho*- and *para*-DTB-lipid compounds were prepared and purified by silica gel chromatography and characterized by NMR and

MALDI-TOFMS, the details of which are given in Example 1.

Fig. 3 shows the mechanism of thiolytic cleavage of the mPEG-DTB-DSPE conjugate. Upon cleavage, the phosphatidylethanolamine lipid is regenerated in its natural, unmodified form.

5 Figs. 4A-4B show a reaction scheme for synthesis of mPEG-DTB-DSPE conjugates having an alkyl group adjacent the disulfide linkage, *e.g.*, a more hindered disulfide linkage. As described more fully in Example 2A, mPEG-OH in dichloromethane was reacted with *p*-nitrophenylchloroformate in the presence of triethylamine (TEA) to form mPEG-nitrophenyl carbonate. An amino alcohol, such as 1-amino-2-propanol or 1-amino-
10 2-butanol, in dimethylformamide (DMF) was reacted with the mPEG-nitrophenyl carbonate in the presence of TEA to form a secondary alcohol attached to PEG. The secondary alcohol was then converted to the desired mPEG-DTB-DSPE compound as illustrated in Fig. 4A and detailed in Example 2A.

In this reaction scheme, mPEG-methyl-dithiobenzyl- nitrophenyl chloroformate was
15 reacted with DSPE to form the desired compound. The nitrophenyl chloroformate moiety in the mPEG-methyl-dithiobenzyl-nitrophenyl chloroformate compound acts as a leaving group to yield the desired product upon reaction with a selected lipid. The invention contemplates, in another aspect, a composition that comprises a compound produced by reaction with a compound such as mPEG-methyl-dithiobenzyl- R^3 , where R^3 represents a
20 leaving group joined through a linking moiety to the benzene ring. The leaving group is displaced upon reaction with an amine-containing ligand, such as DSPE, a polypeptide or an amine-containing drug. The leaving group is selected according to the reactivity of the amine in the ligand, and is preferably derived from various acidic alcohols that have a hydroxy- or oxy-containing leaving group. These include chloride, *p*-nitrophenol, *o*-
25 nitrophenol, N-hydroxy-tetrahydrophthalimide, N-hydroxysuccinimide, N-hydroxy-glutarimide, N-hydroxynorbornene-2,3-dicarboxyimide, 1-hydroxybenzotriazole, 3-hydroxypyridine, 4- hydroxypyridine, 2-hydroxypyridine, 1-hydroxy-6-trifluoromethylbenzotriazole, imidazole, triazole, N-methyl-imidazole, pentafluorophenol, trifluorophenol and trichlorophenol.

30 Example 2B describes preparation of an mPEG-EtDTB-lipid conjugate where the disulfide linkage is hindered by an ethyl moiety.

Fig. 5 shows another synthetic reaction scheme for preparation of an mPEG-DTB-ligand compound in accord with the invention. The details of the reaction procedure are given in Examples 3A-3B. Briefly, cold 1-amino-2-propanol was reacted with sulfuric acid to form 2-amino-1-methylethyl hydrogen sulfate. This product was
5 reacted with carbon disulfide and sodium hydroxide in aqueous ethanol to yield 5-methylthiazolidine-2-thione. An aqueous solution of hydrochloric acid was added to the 5-methylthiazolidine-2-thione and heated. After refluxing for one week, the product, 1-mercapto(methyl)ethyl ammonium chloride, was crystallized and recovered. This product was reacted with methoxy carbonylsulfonyl chloride to yield 2-
10 (methoxycarbonyldithio)ethaneamine. Reaction of the 2-(methoxycarbonyldithio)ethaneamine with mPEG-chloroformate using the procedure described above with respect to Fig. 2 yields the desired mPEG-DTB-nitrophenyl compound suitable for reaction with a selected amine-containing ligand to form a compound in accord with the invention.

15 Example 3B describes the reaction for synthesis of mPEG-(ethyl)DTB-nitrophenyl.

Fig. 6A shows a reaction scheme for preparation of another mPEG-DTB-lipid compound in accord with the invention. The reaction details are provided in Example 4. The lipid 1,2-distearoyl-sn-glycerol is activated for reaction with mPEG-DTB-nitrophenyl,
20 prepared as described in Fig. 4A or Fig. 5. The resulting mPEG-DTB-lipid differs from the compounds described above in the absence of a phosphate head group. The mPEG-DTB-lipid of Fig. 6A is neutral prior to cleavage. As shown in Fig. 6B, upon thiolytic reduction of the disulfide bond, the compound decomposes to yield a cationic lipid. The positively-charged lipid provides for electrostatic interaction *in vivo* and commensurate
25 advantages in *in vivo* targeting.

In the reaction schemes described above, R⁵ of the claimed compound is H. However, in other embodiments R⁵ is an alkyl or an aryl moiety. In this approach, for example where R² and R⁵ are both CH₃ moieties, an α,β -unsaturated acyl chloride (R'R''C=CHCOCl, where R' is, for example CH₃ and R'' is CH₃, however any alkyl
30 or aryl is contemplated) is reacted with an amine-terminated PEG to give the corresponding N-PEG-substituted α,β -unsaturated amide. This compound is reacted

with thiolacetic acid, giving the corresponding N-PEG-substituted β -(acetylthio) amide via conjugate addition to the C=C bond. The acetylthio group (-SCOCH₃) is hydrolyzed to a thiol group (-SH), which is then reacted with methyl (chlorosulfonyl)formate (ClSCOOCH₃), generating a methoxycarbonyl dithio group (-SSCOOCH₃); this intermediate is then reacted with p-mercapto benzyl alcohol to give the N-PEG-substituted β -(dithiobenzyl alcohol) amide (having the structure PEG-NH-CO-CH₂CR'R"-SS-p-phenyl-CH₂OH). The benzyl alcohol moiety is then reacted with nitrophenyl chloroformate to give the nitrophenyl carbonate leaving group, as above.

10 1. In vitro Cleavage of mPEG-DTB-DSPE Compound

The *in vitro* rate of cleavage of *ortho*-mPEG-DTB-DSPE and *para*-mPEG-DTB-DSPE (prepared as described in Example 1) was studied by preparing micellar solutions of the compounds in a buffered aqueous solution (pH 7.2). Thiolytic cleavage of the compounds was monitored in the presence and absence of 150 μ M cysteine by analyzing for disappearance of the compounds by HPLC, as described in Example 5. The results are illustrated in Fig. 7A where the *ortho*- and *para*-compounds in the absence of cysteine (* symbols and + symbols, respectively) show no cleavage and are stable under these conditions in the absence of cysteine. The *ortho*- and *para*-compounds, represented by the open circles and the open squares, respectively, in the presence of 150 μ M cysteine cleave as shown in Fig. 7A. The *ortho*-compound exhibited a slightly faster rate of decomposition than its *para* counterpart ($T_{1/2}$ \approx 12 minutes vs. \approx 18 minutes).

2. Liposome Compositions Comprising an mPEG-DTB-lipid Compound

25 a. In vitro Characterization

In one embodiment, the mPEG-DTB-lipid compound is formulated into liposomes. Liposomes are closed lipid vesicles used for a variety of therapeutic purposes, and in particular, for carrying therapeutic agents to a target region or cell by systemic administration of liposomes. In particular, liposomes having a surface coating of hydrophilic polymer chains, such as polyethylene glycol (PEG), are desirable as drug carries, since these liposomes offer an extended blood circulation lifetime over

liposomes lacking the polymer coating. The polymer chains in the polymer coating shield the liposomes and form a "stiff brush" of water solvated polymer chains about the liposomes. Thus, the polymer acts as a barrier to blood proteins, preventing binding of the protein and recognition of the liposomes for uptake and removal by macrophages and other cells of the reticuloendothelial system.

Typically, liposomes having a surface coating of polymer chains are prepared by including in the lipid mixture between about 1 to about 20 mole percent of the lipid derivatized with the polymer. The actual amount of polymer derivatized lipid can be higher or lower depending on the molecular weight of the polymer. In the present invention, liposomes are prepared by adding between about 1 to about 20 mole percent of the polymer-DTB-lipid conjugate to other liposome lipid bilayer components. As will be demonstrated in the studies described below, liposomes containing the polymer-DTB-lipid conjugate of the invention have a blood circulation lifetime that is longer than liposomes containing a polymer-lipid conjugate where the polymer and lipid are joined by a cleavable aliphatic disulfide bond.

In studies performed in support of the invention, liposomes comprised of the vesicle-forming lipid partially hydrogenated phosphatidyl choline along with cholesterol and the *ortho*-mPEG-DTB-DSPE or the *para*-mPEG-DTB-DSPE compound were prepared as described in Example 6. Cysteine-mediated cleavage of the mPEG-DTB-DSPE compounds was monitored in the presence and absence of 150 μ M cysteine in an aqueous buffer. The results are shown in Fig. 7B, which includes the data of Fig. 7A for comparison. In Fig. 7B, the *ortho*- and *para*-compounds in micellar form in the absence of cysteine (* symbols and + symbols, respectively) show no cleavage, which indicates stability of the conjugate in the absence of thiols. The open circles and the open squares correspond to the *ortho*- and *para*-compounds, respectively, in micellar form in the presence of cysteine, as discussed above with respect to Fig. 7A. The solid circles and the solid squares correspond to the *ortho*- and *para*-compounds, respectively, in liposomal form in the presence of cysteine.

The data in Fig. 7B shows that both the *ortho* and *para* compound were slightly more resistant to thiolytic cleavage when incorporated into liposomes. Examination of the thiolysis reaction products by TLC (silica gel G, chloroform / methanol / water

90:18:2) (Dittmer, J.C., *et al.*, *J. Lipid Res.* 5:126-127 (1964)) showed DSPE as the sole lipid component and another spot corresponding to a thiol-bearing, lipid-free mPEG derivative.

In another study performed in support of the invention, liposomes were prepared from the lipid dioleoyl phosphatidylethanolamine (DOPE) and either the *ortho*-mPEG-DTB-DSPE or the *para*-mPEG-DTB-DSPE compound were prepared. DOPE is a hexagonal phase lipid which alone does not form lipid vesicles. However, liposomes will form when DOPE is combined with a few mole percent of the mPEG-DTB-DSPE compound. Cleavage of the mPEG-DTB-DSPE compound triggers decomposition of the liposomes and release of liposomally-entrapped contents. Thus, the content release characteristics of such liposomes provides for a convenient quantitative evaluation of cleavable PEG-bearing liposomes.

Liposomes comprised of DOPE and the *ortho*- or *para*-mPEG-DTB-DSPE compound were prepared as described in Example 7A with entrapped fluorophores, *p*-xylene-bis-pyridinium bromide and trisodium 8-hydroxypyrenetrisulfonate. Release of the fluorophores from liposomes incubated in the presence of cysteine at various concentrations was monitored as described in Example 7B.

Results for liposomes comprising the *ortho*-compound are shown in Fig. 8A, where percentage of content release of entrapped fluorophore from liposomes incubated in the presence of cysteine at concentrations of 15 μ M (solid diamonds), 150 μ M (solid inverted triangles), 300 μ M (solid triangles) and 1.5 mM (solid circles) are shown. Fig. 8B is a similar plot for liposomes comprising the *para*-compound, where the liposomes are incubated in cysteine at concentrations of 15 μ M (solid diamonds), 300 μ M (solid triangles), 1 μ M (solid squares) and 1.5 mM (solid circles).

Figs. 8A-8B show that both the *ortho*- and *para*-compounds when incorporated into liposome are cleaved, as evidenced by release of the entrapped dye, at a rate dependent on the concentration of cysteine. Control studies with non-cleavable mPEG-DSPE containing liposomes produced no content release (results not shown here). These results also suggest that the *ortho* conjugate is somewhat more susceptible to thiolytic cleavage. For example, 300 μ M cysteine liberates most of the contents of DOPE liposomes within 20 minutes. Under the same conditions, only a fraction of

liposomes having *para*-mPEG-DTB-DSPE decomposed. Similarly, after incubation for 20 minutes at 150 μ M cysteine, half of the entrapped contents was released for the *ortho*-containing liposomes, while only approximately 10% of the contents were released in liposomes containing the *para*-compound. Both *ortho* and *para* compounds have half-lives of less than 20 minutes at a cysteine level of 150 μ M (see data in Fig. 7B). This suggests that more than half of the original three mole percent of the mPEG-DTB-lipid must be cleaved to observe content release from the liposomes.

Decomposition of the mPEG-DTB-DSPE/DOPE liposomes in 15 μ M cysteine, the average plasma concentration in both humans and rodents (Lash, L.H., *et al.*, *Arch. Biochem. Biophys.* 240:583-592 (1985)), was minimal in the time frame of these experiments (60 minutes). This suggests that the mPEG-DTB-lipid compounds should have sufficiently long lifetimes in plasma to allow the PEG-grafted vesicles to distribute systemically *in vivo*, or to accumulate in a specific site either passively or through ligand-mediated targeting. Local or short term increase in cysteine concentration can potentially be achieved by its intravenous or intra-arterial administration. The results shown in Figs. 8A-8B also suggest that a prolonged exposure to the natural plasma cysteine concentration (\approx 15 μ M) would be sufficient to decompose most of these compounds. These suggestions were studied in *in vivo* experiments, described below.

In another study performed in support of the invention, liposomes comprised of DOPE and three different mPEG-DTB-lipid compounds were prepared. The liposomes were prepared as described in Example 7 and included and entrapped fluorophore. The three mPEG-DTB-lipid compounds were mPEG-DTB-DSPE as shown in Fig. 1A; mPEG-MeDTB-DSPE as shown in Fig. 4B, where R is CH₃, and mPEG-MeDTB-distearoyl-glycerol, as shown in Fig. 6A. The liposomes were comprised of 97 mole percent DOPE and 3 mole percent of one of the mPEG-DTB-lipid compounds. Cysteine-mediated rate of cleavage of the compounds was determined by monitoring the release of entrapped fluorophore as a function of time in the presence of various cysteine concentrations. The results are shown in Figs. 9A-9C where the percent release of entrapped fluorophore is normalized for the release rate from liposomes incubated in buffer alone.

Fig. 9A shows the percent release of entrapped fluorophore as a function of time for liposomes comprised of DOPE and *para*-mPEG-DTB-DSPE (compound of Fig. 1A). The

release rate from liposomes containing the conjugate and incubated in the presence of cysteine at concentrations of 15 μ M (solid squares), 75 μ M (open triangles), 150 μ M (X symbols), 300 μ M (open circles), 1500 μ M (solid circles), 3000 μ M (+ symbols), and 15000 μ M (open diamonds) is shown.

5 Fig. 9B shows the percent release of entrapped fluorophore as a function of time for liposomes comprised of DOPE and *para* mPEG-MeDTB-DSPE (compound of Fig. 4B). The release rate of the fluorophore from liposomes incubated in the presence of cysteine at concentrations of 15 μ M (solid squares), 75 μ M (open triangles), 150 μ M (X symbols), 300 μ M (open circles), 1500 μ M (solid circles), 3000 μ M (+ symbols), and 15000 μ M
10 (open diamonds) is shown.

Fig. 9C is a similar plot for liposomes formed with DOPE and mPEG-MeDTB-distearoyl glycerol (compound of Fig. 6A). The release rate of dye from liposomes incubated in the presence of cysteine at concentrations of 15 μ M (solid squares), 75 μ M (open triangles), 150 μ M (X symbols), 300 μ M (open circles), 1500 μ M (solid circles),
15 3000 μ M (+ symbols), and 15000 μ M (open diamonds) is shown.

Figs. 9A-9C show that the rate of mPEG-MeDTB-lipid cleavage is cysteine-concentration dependent, with a slow rate of cleavage, as evidenced by release of entrapped fluorophore, at cysteine concentrations of 15-75 μ M. In comparing the data in Fig. 9A with that in Fig. 9B, it is seen that the mPEG-MeDTB-DSPE compound (Fig. 9B) cleaves approximately 10 times more slowly than the mPEG-DTB-DSPE compound (Fig. 9A). Thus, the rate of cleavage can be tailored according to the R moiety (see Fig. 2) in the DTB linkage.

b. In vivo Characterization

25 The blood circulation lifetime of liposomes prepared as described in Example 8 and that include a polymer-DTB-lipid conjugate in accord with the invention was determined in mice. In¹¹¹ was entrapped in the liposomes and the liposomes were administered by intravenous injection. One group of test animals additionally received an injection of cysteine, the control group of animals additionally received an injection
30 of saline. Blood samples were taken at various times and analyzed for the presence of liposomes, as evidenced by the presence of In¹¹¹.

Fig. 10 shows the results where the counts per minute (CPM) of In^{111} is shown as a function of time following injection of the liposomes and saline (open circles) or 200 mM cysteine (solid squares). As seen, the cleavage of the mPEG-DTB-DSPE occurred upon exposure to the naturally-occurring physiologic conditions, as evidenced by the cleavage in the group of mice treated with saline after administration of the liposomes. Administration of an exogenous reducing agent, cysteine, to the mice was effective to increase the rate of cleavage of the mPEG-DTB-lipid compound in the time frame from between about 2 hours to about 8 hours.

Importantly, cleavage of the polymer-DTB-lipid compound of the invention results in regeneration of the original lipid in unmodified form. This is desirable since unnatural, modified lipids can have undesirable *in vivo* effects. At the same time, the compound is stable when stored in the absence of reducing agents.

In other studies, not shown here, the blood circulation lifetime of liposomes containing the mPEG-DTB-lipid were compared to liposomes containing a polymer-lipid conjugate where the polymer and lipid are joined by a cleavable aliphatic disulfide bond. Aliphatic disulfide linkages are readily cleaved *in vivo* and the blood circulation lifetime of liposomes having polymer chains grafted to their surface by an aliphatic disulfide typically do not have the extended blood circulation lifetime observed for liposomes having stably linked polymer chains. The dithiolbenzyl linkage of the invention, and in particular the more hindered DTB linkages, are more stable *in vivo* and achieve a longer blood circulation lifetime than liposomes with polymer chains attached via an aliphatic disulfide linkage.

B. Amine-Containing Polypeptide

In another embodiment, the invention includes a compound as described with respect to Fig. 1A, where the amine-containing ligand is a polypeptide. A synthetic reaction scheme showing preparation of a polymer-DTB-polypeptide is shown in Fig. 11A, with mPEG as the exemplary polymer. In general, a mPEG-DTB-leaving group compound is prepared according to one the synthetic routes described above in Figs. 2, 4A and 5. The leaving group can be nitrophenyl carbonate or any one of the others described above. The mPEG-DTB-nitrophenyl carbonate compound is coupled to an amine moiety in a

polypeptide by a urethane linkage. The R group adjacent the disulfide in the compound can be H, CH₃, C₂H₅ or the like and is selected according to the desired rate of disulfide cleavage.

Fig. 11B shows the decomposition products upon cysteine-mediated cleavage of the compound. As seen the native protein with no modification to the protein amine group is regenerated upon cleavage.

Attachment of polymer chains, such as PEG, to a polypeptide often diminishes the enzymatic or other biological activity, *e.g.*, receptor binding, of the polypeptide. However, polymer modification of a polypeptide increases the blood circulation lifetime of the polypeptide. In the present invention, the polymer-modified polypeptide is administered to a subject. As the polymer-modified polypeptide circulates exposure to physiologic reducing conditions, such as blood cysteine and other *in vivo* thiols, initiates cleavage of the polymer chains from the polypeptide. As the polymer chains are released from the polypeptide, the biological activity of the polypeptide is gradually restored. In this way, the polypeptide initially has a sufficient blood circulation lifetime for biodistribution, and over time regains its full biological activity as the polymer chains are cleaved.

In a study performed in support of the invention, lysozyme was used as a model polypeptide and an mPEG-MeDTB-lysozyme conjugate was prepared by a synthetic route similar to those described above. Lysozyme was incubated with mPEG-MeDTB-nitrophenylcarbonate in 0.1 M borate, at pH 9 at a 2:1 ratio of nitrophenylcarbonate to amino group of lysozyme. After reaction times of 15 minutes and 3 hours, samples were characterized by SDS-PAGE. A comparative compound was prepared by reacting lysozyme under the same conditions for 60 minutes with a conjugate of mPEG-nitrophenyl carbonate, which will form a stable mPEG-lysozyme conjugate.

Fig. 12 shows a rendering of the SDS-PAGE gel. Lane 1 corresponds to the compound formed after 15 minutes reaction of lysozyme with mPEG-MeDTB-nitrophenylcarbonate and Lane 2 represents the compound formed after a 1 hour reaction time of the same compounds. Lane 3 represents native lysozyme and Lane 4 corresponds to lysozyme reacted for 1 hour with mPEG-nitrophenylcarbonate. The molecular weight markers in Lane 5 are as follows, from the top down:

<u>Molecular Weight (kDaltons)</u>	<u>Marker</u>
1163	β -galactosidase
97.4	phosphorylase b
66.3	bovine serum albumin
55.4	glutamic dehydrogenase
36.5	lactate dehydrogenase
31	carbonic anhydrase
21.5	trypsin inhibitor
14.4	lysozyme

Comparison of Lane 1 and Lane 2 shows that the longer reaction time results in an increase in compound molecular weight, consistent with additional mPEG chains conjugated to the polypeptide at longer incubation time.

5 Lanes 6-9 of the SDS-PAGE profile correspond to the samples in Lanes 1-4 after treatment with 2% β -mercaptoethanol for 10 minutes at 70°C. The mPEG-MeDTB-lysozyme conjugate after exposure to a reducing agent decomposed to regenerate native lysozyme, as evidenced by the band in Lanes 6 and 7 at 14.4 kDa. In contrast, the stable mPEG-lysozyme compound was not affected upon incubation with a reducing agent, as evidenced by the agreement in the profile in Lane 9 and Lane 4.

10 Also evident from the SDS-PAGE profile is that covalent attachment of mPEG-MeDTB to a protein forms a mixture of conjugates containing various mPEG-protein ratios. This ratio is dependent on the reaction time and conditions. This is clearly seen in viewing the bands in Lanes 1 and 2, where Lane 1 shows lysozyme derivatized with from about 1-6 PEG chains. In Lane 2, the longer reaction time yielded mPEG-MeDTB-lysozyme conjugates with a higher mPEG-protein ratio. All cleavable conjugates were readily cleaved to regenerate the native protein, as seen in the bands of Lanes 6 and 7.

20 It will be appreciated that any of the hydrophilic polymers described above are contemplated for use. The molecular weight of the polymer is selected depending on the polypeptide, the number of reactive amines on the polypeptide and the desired size of the polymer-modified compound.

Polypeptides contemplated for use are unlimited and can be naturally-occurring or recombinantly produced polypeptides. Small, human recombinant polypeptides are preferred, and polypeptides in the range of 10-30 KDa are preferred. Exemplary polypeptides include cytokines, such as tumor necrosis factor (TNF), interleukins and
5 interferons, erythropoietin (EPO), granulocyte colony stimulating factor (GCSF), enzymes, and the like. Viral polypeptides are also contemplated, where the surface of a virus is modified to include one or more polymer chain linked via a DTB reversible linkage. Modification of a virus containing a gene for cell transfection would extend the circulation time of the virus and reduce its immunogenicity, thereby improving delivery of
10 an exogeneous gene.

C. Amine-Containing Drug

In yet another embodiment of the invention, a compound of the form polymer-DTB-amine-containing drug is contemplated. The compound is of the structure described
15 above, and in particular with respect to Fig. 1A where the amine-containing ligand in the figure is the amine-containing drug. Modification of therapeutic drugs with PEG is effective to improve the blood circulation lifetime of the drug and to reduce any immunogenicity.

A polymer-DTB-amine-containing drug is prepared according to any of the reaction
20 schemes described above, with modifications as necessary to provide for the particular drug. A wide variety of therapeutic drugs have a reactive amine moiety, such as mitomycin C, bleomycin, doxorubicin and ciprofloxacin, and the invention contemplates any of these drugs with no limitation. It will be appreciated that the invention is also useful for drugs containing an alcohol or carboxyl moiety. In the case where the drug
25 contains a hydroxyl or carboxyl moiety suitable for reaction, the polymer-DTB moiety can be linked to the drug via urethane, ester, ether, thioether or thioester linkages. In all of these embodiments, the polymer-DTB-drug compound after administration *in vivo*, thiolytically decomposes to regenerate the amine-containing drug in its native, active form, therapeutic activity of the compound after modification and prior to administration is not
30 necessary. Thus, in cases where modification of the drug with the DTB-polymer causes a reduction or loss of therapeutic activity, after administration and cleavage of the DTB-

polymer from the drug, activity of the drug is regained.

In studies performed in support of the invention, the drug nitroanilide was reacted with mPEG-MeDTB-nitrophenylcarbonate to form an mPEG-MeDTB-*para*-nitroanilide compound, as shown in Fig. 13. Decomposition of the compound upon exposure to a
5 reducing agent yields the products shown in the figure, with the drug *para*-nitroanilide regenerated in an unmodified state.

The mPEG-MeDTB-*para*-nitroanilide compound was incubated *in vitro* in buffer containing 5 mM cysteine and the absorbance of samples withdrawn at various times is shown in Fig. 14A. Seen in the figure are samples measured at the following time
10 points: time zero (closed diamonds), 2 minutes (closed squares), 5 minutes (x symbols), 10 minutes (open squares), 20 minutes (triangles), 40 minutes (open diamonds) and 80 minutes (closed circles). The change in the UV spectra as a function of incubation time in cysteine is evident, showing cysteine-mediated release of *para*-nitroanilide from the mPEG-MeDTB- *para*-nitroanilide compound.

Fig. 14B shows the amount of *para*-nitroanilide, in mole/L, released *in vitro* from
15 the mPEG-MeDTB-*para*-nitroanilide conjugate incubated in the presence of 5 mM cysteine (closed circles), 1 mM cysteine (closed squares) and 0.15 mM cysteine (closed diamonds). The rate of drug release from the conjugate was dependent on the concentration of reducing agent present.

From the foregoing, it can be seen how various objects and features of the invention
20 are met. The compounds of the invention comprise an amine-containing ligand reversibly joined to a hydrophilic polymer via an ortho or para-disulfide of a benzyl urethane linkage. This linkage when subjected to mild thiolytic conditions is cleaved to regenerate the original amine-containing ligand in its unmodified form. The rate of cleavage can be
25 controlled by steric hinderance of the disulfide in the linkage and/or by controlling the thiolytic conditions *in vivo*. The compounds prior to cleavage of the dithiobenzyl linkage are provided with an increased blood circulation lifetime, improved stability and reduced immunogenicity.

III. Examples

The following examples further illustrate the invention described herein and are in no way intended to limit the scope of the invention.

5 Materials

All materials were obtained from commercially suitable vendors, such as Aldrich Corporation.

Example 1 10 Synthesis of mPEG-DTB-DSPE

mPEG-MeDTB-nitrophenylcarbonate (300 mg, 0.12 mmol, 1.29 eq) was dissolved in CHCl_3 (3 ml). DSPE (70 mg, 0.093 mol) and TEA (58.5 μl , 0.42 mmol, 4.5 eq) were added to PEG-solution, and was stirred at 50°C (oil bath temp). After 15 minutes, TLC showed that the reaction didn't go to completion. Then two portions of TEA (10 μl , and 20 μl), and few portions of mPEG-MeDTB-nitrophenylcarbonate (50 mg, 30 mg, 10 mg) were added every after 10 minutes, until the reaction went to completion. Solvent was evaporated. Product mixture was dissolved in MeOH, and 1 g of C8 silica was added. Solvent was evaporated again. Product containing C8 silica was added on the top of the column, and was eluted with MeOH: H_2O gradient (pressure), MeOH: H_2O = 30: 70, 60 ml; MeOH: H_2O = 50: 50, 60 ml; MeOH: H_2O = 70: 30, 140 ml (starting material eluted); MeOH: H_2O = 75: 25 = 40 ml; MeOH: H_2O = 80: 20, 80 ml (product eluted); MeOH: H_2O = 85: 15, 40 ml; MeOH: H_2O = 90: 10, 40 ml; MeOH = 40 ml; CHCl_3 : MeOH: H_2O = 90: 18: 10, 40 ml. Fractions containing pure product were combined and evaporated to give product as colorless thick liquid. Tertiary butanol (5 ml) was added to it, lyophilized and the dried in *vacuo* over P_2O_5 to give product as white fluffy solid (252 mg, 89% yield).

The *ortho*- and *para*-DTB-DSPE compounds were purified by silica gel chromatography (methanol gradient 0-10% in chloroform, $\approx 70\%$ isolated yield) and the structures confirmed by NMR and MALDI-TOFMS. (^1H NMR for *para* conjugate: (d6-DMSO, 360 MHz) δ 0.86 (t, CH_3 , 6 H), 1.22 (s, CH_2 of lipid, 56H), 1.57 (m, $\text{CH}_2\text{CH}_2\text{CO}_2$, 4H), 2.50 (2xt, CH_2CO_2 , 4H), 2.82 (t, CH_2S , 2H), 3.32 (s, OCH_3 , 3H),

3.51 (m, PEG, \approx 180 H), 4.07 (t, PEG-CH₂CONH, 2H), 4.11 & 4.28 (2 x dd CH₂CH of glycerol, 2H), 4.98 (s, benzyl-CH₂, 2H), 5.09 (m, CHCH₂ of lipid), 7.35 & 7.53 (2 x d, aromatic, 4H) ppm. The *ortho* conjugate differed only in benzyl and aromatic signals at 5.11 (s, CH₂, 2H), and 7.31 (d, 1H), 7.39 (m, 2H) 7.75(d, 1H) ppm.

5 MALDI-TOFMS produced a distribution of ions spaced at equal 44 Da intervals, corresponding to the ethylene oxide repeating units. The average molecular weights of the compounds was 3127 and 3139 Da for *para* and *ortho* isomers respectively (theoretical molecular weight \approx 3100 Da).

The reaction scheme is illustrated in Fig. 2.

Example 2

Synthesis of mPEG-DTB-DSPE

A. mPEG-MeDTB-DSPE

This reaction scheme is illustrated in Figs. 4A-4B.

15 mPEG(5K)-OH (40 g, 8 mmol) was dried azeotropically with toluene (total volume was 270 ml, 250 ml was distilled off by Dean-Stark). Dichloromethane (100 ml) was added to mPEG-OH. P-nitrophenyl chloroformate (2.42 g, 12 mmol, 1.5 eq), and TEA (3.3 ml, 24 mmol, 3 eq) were added to PEG solution at 4°C (ice water), while taking precautions against moisture. Light yellow TEA hydrochloride salt was formed. 20 After 15 minutes cooling bath was removed, and the reaction mixture was stirred at room temperature overnight. TLC showed (CHCl₃: MeOH: H₂O = 90: 18: 2) that the reaction was complete. Solvent was evaporated. The residue was dissolved in ethyl acetate (\sim 50°C). TEA hydrochloride salt was filtered off and washed with warm ethyl acetate. Solvent was evaporated and the product recrystallized with isopropanol (three 25 times). Yield: 38.2 g (92%). ¹H NMR (DMSO-*d*₆, 360 MHz) δ 3.55 (s, PEG, 450H); 4.37 (t, PEG-CH₂, 2H); 7.55 (d, C₆H₅, 2H); 8.31 (d, C₆H₅, 2H).

1-Amino-2-propanol (1.1 ml, 14.52 mmol, 3 eq), and TEA (2.02 ml, 14.52 mmol, 3 eq) were added to mPEG (5K)-nitrophenyl carbonate (25 g, 4.84 mmol) in DMF (60 ml) and CH₂Cl₂ (40 ml). It was a yellow clear solution. The reaction 30 mixture was stirred at room temperature for 30 minutes. TLC (CHCl₃: MeOH = 90: 10) showed that the reaction went to completion. Solvent (dichloromethane) was

evaporated. Isopropanol (250 ml) was added to the product mixture in DMF (60 ml). Product precipitated immediately, and then recrystallized with iPrOH (three times). Yield: 22.12 g (90%). ¹H NMR (DMSO-*d*₆, 360 MHz) δ .98 (d, CH₃CH(OH)CH₂, 3H); 3.50 (s, PEG, 180H); 4.03 (t, PEG-CH₂, 2H); 4.50 (d, CH₃CHOH, 1H); 7.0 (t, mPEG-OCONH).

mPEG(5K)-urethane-2-methyl propanol (22.12 g, 4.34 mmol) was dried azeotropically with toluene (45 ml). Dichloromethane (60 ml) was added to it. Methane sulfonyl chloride (604.6 μl, 7.81 mmol, 1.8 eq) and TEA (3.93 ml, 28.21 mmol, 6.5 eq) were added to mPEG-solution at 0°C while maintaining stirring and taking precautions against moisture. After 30 minutes, cooling bath was removed, and the reaction mixture was stirred at room temperature for 16 h. Solvent was evaporated. Ethyl acetate was added to remove TEA salts. The product was recrystallized with isopropanol (three times). Yield: 20.27 g (90%). ¹H NMR (DMSO-*d*₆, 360 MHz) δ 1.27 (d, CH₃CHOSO₂CH₃, 3H); 3.162 (s, CH₃O₂SOCH, 3H); 3.50 (s, PEG, 180H); 4.07 (t, PEG-CH₂, 2H); 4.64 (q, CH₃CHOH, 1H); 7.43 (t, mPEG-OCONH).

mPEG(5K)-urethane-2methyl-methane sulfone (10.27 g, 1.98 mmol) was dried azeotropically with toluene (20 ml, each time). Sodium hydride (377 mg, 9.4 mmol, 4.75 eq) was added in anhydrous toluene (60 ml) at 0°C (in ice water). After 5 minutes, triphenylmethanethiol (3.92 g, 14.6 mmol, 7.15 eq) was added to the solution. After 10 minutes, mPEG-urethane-2methyl-methane sulfone (10.27 gm, 1.98 mmol) was added to the reaction mixture. It became a yellow solution. After 45 minutes, TLC (CHCl₃: MeOH: H₂O = 90: 18: 2) showed that the reaction went to completion. Acetic acid (445.57 μl, 7.42 mmol, 3.75 eq) was added to the reaction mixture to neutralize excess of sodium hydride. The solution became thick and whitish. Solvent was evaporated and the solid was recrystallized with ethyl acetate (30 ml) and isopropanol (70ml). The product mixture did not dissolve completely, while precipitate filtered off. Then the product mixture was recrystallized with isopropanol/tert-butyl alcohol (100ml/20ml). Yield: 8.87 g (84%). ¹H NMR (DMSO-*d*₆, 360 MHz) δ .74 (d, CH₃CHSC(C₆H₅)₃, 3H), 3.50 (s, PEG, 180H), 4.0 (t, PEG-CH₂, 2H), 4.64 (q, CH₃CHOH, 1H); 7.49 (t, mPEG-OCONH); 7.20-7.41 (m, SC(C₆H₅)₃, 15H).

mPEG(5K)-urethane-2methyl-triphenylmethanethiol (8.87 g, 1.65 mmol) was

dissolved in TFA/CH₂Cl₂ (10ml/10ml) at 0°C. Under vigorous stirring, methoxy carbonylsulfonyl chloride (185.5 µl, 1.99 mmol, 1.2 eq) was added to the solution. The reaction mixture was stirred at room temperature for 15 minutes. TLC (CHCl₃: MeOH = 90: 10) showed that the reaction was complete. Solvents were evaporated. The product mixture was recrystallized with isopropanol:tert-butyl alcohol (80 ml: 20 ml) two times. Tertiary butanol (5 ml) was added to the product, which was then lyophilized and dried in *vacuo* over P₂O₅ to give product as white fluffy solid (8.32g, 97% yield). ¹H NMR (DMSO-*d*₆, 360 MHz) δ 1.17 (d, CH₃CHSSCOOCH₃, 3H); 3.42 (s, PEG, 180H); 3.84 (s, CH₃OCOSSH, 3H); 4.05 (t, mPEG-CH₂, 2H); 7.38 (t, mPEG-OCONH, 1H).

mPEG(5K)-urethane ethyl(methyl)dithiocarbonyl methoxide (8.32 g, 1.6 mmol) was dissolved in dry methanol (20 ml), and chloroform (2.5 ml). A solution of mercapto benzyl alcohol (592 mg, 4 mmol, 2.5 eq) in dry methanol (2 ml) was added to the PEG-solution. The reaction mixture was stirred at room temperature for 18 h. Solvent was evaporated, product mixture was recrystallized with ethyl acetate/isopropanol, 30 ml/100 ml (3 times). NMR showed ~16% product was formed. So, another portion of mercapto benzyl alcohol (322 mg, 2.18 mmol, 1.8 eq) in MeOH (2ml) was added dropwise to the product mixture in MeOH/CHCl₃ (24 ml/1 ml) at 0°C (ice water). After addition (~10 minutes) completion, ice bath was removed, and the reaction mixture was stirred at room temperature for 24 h. TLC (CHCl₃: MeOH: H₂O = 90: 18: 2) showed that the reaction was complete. Solvent was evaporated, and then product mixture was recrystallized with ethyl acetate/isopropanol, 30 ml/100 ml. Yield: 7.25 g, (94%). ¹H NMR (DMSO-*d*₆, 360 MHz) δ 1.56 (d, CH₃CHSSC₆H₅CH₂OH, 3H); 3.29 (CH₃O-PEG, 3H); 3.50 (s, PEG, 450H); 4.03 (t, mPEG-CH₂, 2H); 4.46 (d, HOCH₂C₆H₅, 2H); 5.16 (t, HOCH₂C₆H₅, 1H); 7.30 (d, C₆H₅, 2H); 7.40 (br t, mPEG-OCONH, 1H); 7.50 (d, C₆H₅, 2H).

mPEG(5K)-urethane-ethyl(methyl)-dithiobenzyl alcohol (6.75 g, 1.27 mmol) was dissolved in CHCl₃ (30 ml), P-nitrophenyl chloroformate (513 mg, 2.54 mmol, 2 eq) was added to it at 0°C (ice water). After 5 minutes triethylamine (531 µl, 3.81 mmol, 3 eq) was added. After 30 minutes ice bath was removed, and the reaction mixture was stirred at room temperature overnight. Solvent was evaporated. The

product mixture was dissolved in ethyl acetate. TEA salt was filtered off, and then solvent was evaporated. Then the product mixture was recrystallized with ethyl acetate/isopropanol, 30 ml/100 ml (three times). Yield: 6.55 g (94%). ¹H NMR (DMSO-*d*₆, 360 MHz) δ 1.17 (d, CH₃CHSSC₆H₅, 3H); 3.24 (CH₃O-PEG, 3H); 3.40 (s, PEG, 180H); 4.03 (br t, mPEG-CH₂, 2H); 5.28 (s, C₆H₅CH₂OCO, 2H); 7.45-8.35 (m, C₆H₅)₂, 8H)

mPEG-MeDTB-nitrophenylcarbonate (766 mg, 0.14 mmol, 1.29 eq) was dissolved in CHCl₃ (5 ml). DSPE (70 mg, 0.093 mol) and TEA (58.5 μl, 0.42 mmol, 4.5 eq) were added to PEG-solution, and was stirred at 50°C (oil bath temp). After 20 minutes, TLC showed that the reaction didn't go to completion. More mPEG-MeDTB-nitrophenylcarbonate (total 1239 mg, 0.23 mmol, 2.47 eq) and 1-hydroxybenztriazole (HOBt) (25 mg, 0.19 mmol, 2 eq) were added. After 20 minutes, TLC (CHCl₃: MeOH: H₂O = 90: 18: 2, with molybdenum and ninhydrin) showed that the reaction was complete. Solvent was evaporated. Product mixture was dissolved in warm (42°C) ethyl acetate. It was a cloudy solution (TEA salt precipitated). The solution was filtered, and solvent was evaporated. MeOH, and 2 g of C8 silica was added to the product mixture. Solvent was evaporated again. Product containing C8 silica was added on the top of the column, and was eluted with MeOH: H₂O gradient (pressure), MeOH : H₂O 30: 70, 100 ml; MeOH H₂O 50: 50, 100 ml; MeOH H₂O 70: 30, 250 ml (starting material eluted); MeOH H₂O 75: 25 = 40 ml; MeOH H₂O 80: 20, 200 ml (product eluted); MeOH = 100 ml; CHCl₃: MeOH: H₂O = 90: 18: 2, 100 ml; CHCl₃: MeOH H₂O = 75: 36: 6, 100 ml. Fractions containing pure product were combined and evaporated to give product as colorless thick liquid. Tertiary butanol (5 ml) was added to it, lyophilized and then dried in *vacuo* over P₂O₅ to give product as white fluffy solid (467 mg, 83% yield). ¹H NMR (DMSO-*d*₆, 360 MHz) δ 0.83 (d, 2(CH₃), 3H); 1.16 (d, CH₃CHSSC₆H₅, 3H); 1.21 (s, 28(CH₂, 56H); 1.47 (br m, CH₂CH₂CO, 4H); 2.23 (2 x t, CH₂CH₂CO, 4H); 3.50 (s, PEG, 180H); 4.04 (br t, mPEG-CH₂, 2H); 4.05 (trans d, PO₄CH₂CHCH₂, 1H); 4.24 (cis d, PO₄CH₂CHCH₂, 1H); 4.97 (s, C₆H₅CH₂OCO-DSPE, 2H); 5.03 (br s, (PO₄CH₂CH, 1H); 7.32 (d, C₆H₅, 2H); 7.53 (d, C₆H₅, 2H); 7.52 (br s, mPEG-OCONH, 1H). MALDI-TOFMS produced a bell shaped distribution of ions spaced at equal 44 Da intervals, corresponding to the ethylene oxide

repeating units. The average molecular mass of the conjugate and mPEG-thiol (mostly cleaved disulfide) is 6376 and 5368 Da (theoretical molecular mass ~6053, and 5305 Daltons).

5 B. mPEG-ethylDTB-DSPE

mPEG-urethane ethyl(ethyl)dithiocarbonyl methoxide (2 g, 0.90 mmol) was dissolved in dry methanol (8 ml). At the beginning the solution was cloudy, but after 5 minutes it became a clear solution. Mercaptobenzyl alcohol (265.2 mg, 1.79 mmol, 2 eq) was added to the PEG-solution. The reaction mixture was stirred at room
10 temperature for 30 hours. Ether (70 ml) was added to the reaction solution to precipitate the product, and kept at 4°C overnight. The white solid was filtered and recrystallized with ethyl acetate/ether, 30 ml/70 ml. Yield : 1.96 g, (94%). ¹H NMR (DMSO-*d*₆, 360 MHz) δ0.86 (d, CH₃CH₂CHSSC₆H₅CH₂OH, 3H); 1.42 (p, CH₃CH₂CHSSC₆H₅CH₂OH, 1H); 1.64 (p, CH₃CH₂CHSSC₆H₅CH₂OH, 1H); 3.51 (s, PEG, 180H); 4.03 (t, mPEG-CH₂, 2H); 4.47 (d, HOCH₂C₆H₅, 2H); 5.20 (t, HOCH₂C₆H₅, 1H); 7.31(d, C₆H₅, 2H); 7.42 (br t, mPEG-OCONH, 1H); 7.49 (d, C₆H₅, 2H).
15

N-hydroxy-s-norbornene-2,3-dicarboxylic acid imide (HONB) (48 mg, 0.269 mmol) was added to DSPE (55 mg, 0.073 mmol) in CHCl₃ (3 ml) at 50°C (oil bath
20 temperature). After 3-4 minutes it became a clear solution. Then mPEG-EtDTB-nitrophenylchloroformate (334 mg, 0.134 mmol) was added, followed by triethylamine (TEA, 45 μl, 0.329 mmol). After 20 minutes TLC (CHCl₃ : MeOH : H₂O = 90 : 18 : 2) showed that the reaction went to completion (molybdenum and ninhydrin sprays). Solvent was evaporated. Product mixture was dissolved in methanol, mixed with C8
25 silica (1g) and stripped of the solvent by rotary evaporation. The solid residue was added on the top of the C8-column, which was then eluted with MeOH : H₂O gradient (pressure), MeOH : H₂O = 30 : 70, 60 ml; MeOH : H₂O = 50 : 50, 60 ml; MeOH : H₂O = 70 : 30, 140 ml; MeOH : H₂O = 75 : 25 = 140 ml (starting material eluted); MeOH : H₂O = 80 : 20, 80 ml; MeOH : H₂O = 90 : 10, 140 ml (product eluted);
30 MeOH = 40 ml; CHCl₃ : MeOH : H₂O = 90 : 18 : 10, 40 ml. Fractions containing pure product were combined and evaporated to give product as colorless thick liquid.

Tertiary butanol (5 ml) was added, lyophilized and then dried in *vacuo* over P_2O_5 to give product as white fluffy solid (175 mg, 78% yield). 1H NMR (DMSO- d_6 , 360 MHz) δ 0.85 (d, 2(CH_3), 6H); d, $CH_3CHSSC_6H_5$, 3H); 1.22 (s, 28(CH_2), 56H); 1.49 (br m, CH_2CH_2CO , 4H); 2.24 (2 x t, CH_2CH_2CO , 4H); 3.50 (s, PEG, 180H); 4.04 (br t, mPEG- CH_2 , 2H); 4.08 (trans d, $PO_4CH_2CHCH_2$, 1H); 4.27 (cis d, $PO_4CH_2CHCH_2$, 1H); 4.98 (s, $C_6H_5CH_2OCO$ -DSPE, 2H); 5.06 (br s, (PO_4CH_2CH , 1H); 7.34 (d, C_6H_5 , 2H); 7.53 (d, C_6H_5 , 2H); 7.55 (br s, mPEG-OCONH, 1H).

Example 3

10 Synthesis of mPEG-DTB-nitrophenylchloroformate

A. Procedures for synthesis of 1-(mercaptomethyl) ethylammonium chloride

1. 2-Amino-1-methylethyl hydrogen sulfate. 1-Amino-2-propanol (22.53g, 0.3 mol) was vigorously stirred in an ice bath. Sulfuric acid (16.10 ml, 0.3 mol) was added very slowly, over the course of one hour. Thick vapors and a very viscous solution were formed in the flask. After addition was complete, the reaction was heated between 170°C and 180°C, under reduced pressure, connected to the house vacuum. Upon heating, the reaction turned light brown. After all water was removed (approximately 1 hour) it was allowed to cool to room temperature. Upon cooling a brown, glassy solid was formed which would crystallize when triturated with methanol. It was dissolved in water (50 ml) at 60°C. Enough warm methanol was added to make the solution 80% methanol. Upon cooling, crystals formed which were then filtered and dried over P_2O_5 . Yield: 17.17g (37 %). 1H NMR (D_6 -DMSO): δ 1.16 (d, CH_3 , 3H); δ 2.78 (dd, NH_3-CH_2 , 1H); δ 2.97 (dd, NH_3-CH_2 , 1H); δ 4.41 (m, $CH-OSO_3$, 1H); δ 7.69 (s, H_3N , 3H). Melting point: 248°-250°C (lit: 250°C)

2. 5-Methylthiazolidine-2-thione. 2-Amino-1-methylethyl hydrogen sulfate (23.03 g, 148 mmol) and carbon disulfide (10.71 ml, 178 mmol, 1.2 eq.) were stirred in a 250 ml round-bottom-flask in 50% aqueous ethanol (40 ml). To this, sodium hydroxide (13.06 g, 327 mmol, 2.2 eq.) in 50% aqueous ethanol (50 ml) was added drop-wise, very slowly. Upon addition of sodium hydroxide, all starting materials dissolved and the solution turned orange. The reaction was refluxed (85°C) for 40 minutes, after which time it turned bright yellow and a thick precipitate was formed.

Ethanol was evaporated and then the aqueous solution was warmed and then filtered through a Buchner funnel to remove all water-soluble impurities. The remaining crystals were dissolved in warm ethanol and then warm water was added until the solution was 80% water. The mixture was allowed to cool and then refrigerated, yielding long, needle-like crystals. Yield: 14.64 g (75%). ¹H NMR (D₆-DMSO): δ 1.33 (d, CH₃, 3H); δ 3.50 (m, R₃CH, 1H); δ 3.95 (dd, N-CH₂, 1H); δ 4.05 (m, N-CH₂, 1H); δ 10.05 (s, NH, 1H). Melting point: 92.5-93.5 (lit: 94-95).

3. 1-(mercaptomethyl)ethylammonium chloride. 5-Methylthiazolidine-2-thione (6.5 g, 49 mmol) was placed in a 250 ml round-bottom-flask. A solution of aqueous hydrochloric acid (40 ml, 18% in H₂O) was added and the flask was heated in an oil bath. The reaction refluxed (120°C) for one week. Three times throughout the week 1 ml of concentrated hydrochloric acid was added. The reaction was monitored using TLC with ethyl acetate as eluent. They were visualized using UV, ninhydrin, and iodine vapors. Through most of the week the reaction was a heterogeneous mixture, with the starting material as oil which was denser than water. After one week the oil starting material was gone, although still visible on TLC. The reaction was removed from heat and allowed to cool to room temperature, and then was refrigerated to crystallize starting material. The crystallized starting material was filtered. Filtrate was evaporated and it was dried over P₂O₅ and NaOH to remove all water and HCl. The crude product was washed with two portions of diethyl ether (50 ml each) to remove all starting material. It was again dried over P₂O₅. Yield: 2.83 g (45%). ¹H NMR (D₆-DMSO): δ 1.33 (d, CH₃, 3H); δ 2.92 (m, N-CH₂, 2H); δ 3.12 (m, SH, 1H); δ 3.18 (m, R₃-CH, 1H); δ 8.23 (bs, NH₃, 3H). Melting point: 80-82°C (lit: 92-94).

The reaction scheme is illustrated in Fig. 5.

B. Synthesis of mPEG-ethyl-DTB-nitrophenylchloroformate

1. 2-Amino-1-ethylethyl hydrogen sulfate. 1-Amino-2-butanol (15 ml, 158 mmol) was vigorously stirred in a 100 ml round-bottom-flask in an ice bath. Sulfuric acid (8.43 ml, 158 mmol) was added very slowly, over the course of one hour. Thick vapors and a very viscous solution were formed in the flask. After addition was

complete, the reaction was heated between 170° and 180°C, under reduced pressure, connected to the house vacuum. Upon heating, the reaction turned light brown. After all water was removed (approximately 1 hour) it was allowed to cool to room temperature. Upon cooling a brown, glassy solid was formed. It was dissolved in hot water (50 ml) and then placed in the refrigerator overnight. Upon cooling, crystals formed which were then filtered and dried over P₂O₅. Yield: 9.98 g (37%). ¹H NMR (D₆-DMSO): δ 0.87 (t, CH₃, 3H); δ 1.51 (q, CH₃-CH₂, 2H); δ 2.82 (dd, NH₃-CH₂, 1H); δ 3.00 (dd, NH₃-CH₂, 1H); δ 4.21 (m, CH-OSO₃, 1H); δ 7.70 (s, H₃N, 3H).

2. 5-Ethylthiazolidine-2-thione. 2-Amino-1-ethyl-ethyl hydrogen sulfate (9.98 g, 59 mmol) and carbon disulfide (4.26ml, 71 mmol, 1.2 eq.) were stirred in a 100 ml round-bottom-flask in 50% aqueous ethanol (15ml). To this, sodium hydroxide (5.20 g, 130 mmol, 2.2 eq.) in 50% aqueous ethanol (20 ml) was added drop-wise, very slowly. Upon addition of sodium hydroxide, all starting materials dissolved and the solution turned orange. The reaction was refluxed (85°C) for 40 minutes, after which time it turned bright yellow and a thick precipitate was formed. Ethanol was evaporated and then the aqueous solution was warmed and then filtered through a Buchner funnel to remove all water-soluble impurities. The remaining crystals were dissolved in warm ethanol and then warm water was added until the solution was 80% water. The mixture was allowed to cool and then refrigerated, yielding needle-like crystals. Yield: 7.28g (86%). ¹H NMR (D₆-DMSO): δ 0.88 (t, CH₃, 3H); δ 1.66 (in, CH₃-CH₂, 2H); δ 3.58 (m, R₃CH, 1H); δ 3.93 (m, N-CH₂, 2H); δ 10.06 (s, NH, 1H). Melting point: 76-78° (lit: 76.6-76.9).

3. 1-(mercaptoethyl)ethylammonium chloride. 5-Ethylthiazolidine-2-thione (7.24 g, 50 mmol) was placed in a 250 ml round-bottom-flask. A solution of aqueous hydrochloric acid (45 ml, 18% in H₂O) was added and the flask was heated in an oil bath. Upon heating, the starting material melted, forming, all heterogeneous mixture. The reaction refluxed (120°C) for one week. Four times throughout the week 1 ml of concentrated hydrochloric acid was added. The reaction was monitored using TLC with ethyl acetate as eluent. They were visualized using UV, ninhydrin, and iodine

vapors. Throughout the week the reaction was a heterogeneous mixture, with the starting material as oil which was denser than water. The reaction was removed from heat and allowed to cool to room temperature, and then was refrigerated to crystallize starting material. The crystallized starting material was filtered. Filtrate was
5 evaporated and it was dried over P_2O_5 and NaOH to remove all water and HCl. The crude product was washed with two portions of diethyl ether (50 ml each) to remove all starting material. It was again dried over P_2O_5 . Yield: 3.66 g (52%). 1H NMR (D_6 -DMSO):

The reaction scheme is illustrated in Fig. 5.

Example 4 Synthesis of mPEG-DTB-lipid

1, 2-distereoyl-sn-glycerol (500 mg, 0.8 mmol) was dried azeotropically with benzene (3 times). *Para*-nitrophenyl chloroformate (242 mg, 1.2 mmol, 1.5 eq),
15 dimethylaminopyridine (DMAP) (10 mg, 0.08 mmol, 0.1 eq), and TEA (334.5 μ l, 2.4 mmol, 3 eq) were added to 1, 2-distereoyl glycerol in $CHCl_3$ (5 ml). The reaction mixture was stirred at room temperature for 2 h. TLC (Toluene: ethyl acetate = 7: 3) showed that the reaction was complete. Then the product mixture was extracted with 10% citric acid to remove dimethylaminopyridine (DMAP), washed with acetonitrile (3
20 ml, 4 times) to remove excess of *p*-nitrophenyl chloroformate. Pure product was dried in *vacuo* over P_2O_5 . Yield: 557 mg(88%). 1H NMR ($CHCl_3$, 360 MHz) δ 0.88 (t, end CH_3 , 6H); 1.25 (s, $28 \times CH_2$, 56H); 1.58 (m, CH_2CH_2CO , 4H); 2.34 (2xt, CH_2CO , 4H); 4.22 (trans d, $CH_2OCOC_{17}H_{35}$, 1H); 4.35 (m, $OCOOCH_2CH$, 2H); 4.51 (cis d, $CH_2OCOC_{17}H_{35}$, 1H); 5.37 (m, $OCOOCH_2CH$, 1H); 7.39 (d, C_6H_5 , 2H); 8.28 (d,
25 C_6H_5 , 2H).

Ethylene diamine (42 μ l, 0.63 mmol, 5 fold excess), and pyridine (200 μ l, were added in $CHCl_3$ (1 ml). 2-distereoyl-sn-*p*-nitrophenyl carbonate (100 mg, 0.13 mmol) was dissolved in $CHCl_3$ (1 ml) and added dropwise to ethylene diamine solution with a pastuer pipette at 0°C (ice water) and continued overnight (16 h). TLC ($CHCl_3$:
30 MeOH: H_2O 90: 18: 2, and $CHCl_3$: MeOH = 90: 10) showed that the reaction was complete. Solvent was evaporated to remove pyridine. Then the product mixture was

dissolved in CHCl_3 , loaded onto the column (Aldrich, Silica gel, 60°A, 200-400 mesh), and eluted with CHCl_3 : CH_3COCH_3 , and CHCl_3 : MeOH gradient, CHCl_3 : CH_3COCH_3 = 90: 10, 60 ml (upper spot eluted); CHCl_3 : MeOH = 90: 10, 60 ml (product eluted). Fractions containing pure product were combined and evaporated.

5 Tert-butanol was added and dried in *vacuo* over P_2O_5 . Yield: 64 mg (75%). ^1H NMR ($\text{DMSO}-d_6$, 360 MHz) δ .83 (t, end CH_3 , 6H); 1.22 (s, $28\times\text{CH}_2$, 56H); 1.51 (m, $\text{CH}_2\text{CH}_2\text{CO}$, 4H); 2.25 (2xt, CH_2CO , 4H); 2.83 (m, $\text{H}_2\text{NCH}_2\text{CH}_2\text{NH}$, 2H); 3.21 (m, $\text{H}_2\text{NCH}_2\text{CH}_2\text{NH}$, 2H); 4.10-4.14 (m & cis d, $\text{COOCH}_2\text{CHCH}_2$, 4H); 5.17 (m, OCOOCH_2CH , 1H); 7.78 (m, $\text{H}_2\text{NCH}_2\text{CH}_2\text{NH}$, 2H).

10 mPEG-MeDTB-nitrophenylchloroformate (400 mg, 0.162 mmol, 2.2 eq) was dissolved in CHCl_3 in (2 ml). 1,2-steroyl-sn-ethylene amine (51 mg, 0.075 mmol) and TEA (37 μl , 0.264 mmol, 3.52 eq) were added to the solution. Then the reaction mixture was stirred at 45°C for 20 minutes. TLC (CHCl_3 : MeOH: H_2O = 90: 18: 2, and CHCl_3 : MeOH = 90: 10) showed that the reaction went to completion. Solvent was evaporated. The product mixture was dissolved in methanol. 2 g of C8 silica was added and then solvent was evaporated. C8 silica containing product mixture was added on the top of the C8 column ((Supelco, Supel clean. Lot no. SP0824), and was eluted with MeOH: H_2O gradient (pressure), MeOH: H_2O = 60: 40, 40 ml; MeOH: H_2O = 70: 30, 80 ml (starting material eluted); MeOH: H_2O = 80: 20, 40 ml; MeOH: H_2O = 90: 10 = 20 ml; CHCl_3 : MeOH: H_2O = 5: 80: 15, 20 ml; CHCl_3 : MeOH: H_2O = 90: 18: 10, 40 ml (product eluted). Fractions containing pure product were combined and evaporated to give product as colorless thick liquid. Tertiary butanol (5 ml) was added and the solution was lyophilized and then dried in *vacuo* over P_2O_5 to give product as white solid (200 mg, 89% yield). ^1H NMR ($\text{DMSO}-d_6$, 360 MHz) δ .83 (t, end CH_3 , 6H); 1.22 (s, $28\times\text{CH}_2$, 56H); 1.48 (m, $\text{CH}_2\text{CH}_2\text{CO}$, 4H); 2.25 (2 x t, CH_2CO , 4H); 3.10 (m, $\text{HNCH}_2\text{CH}_2\text{NH}$, 4H); 3.50 (s, PEG, 180H); 4.04 (t, mPEG- CH_2 , 2H); 4.09 (trans d, $\text{COOCH}_2\text{CHCH}_2$, 1H); 4.25 (cis d, $\text{COOCH}_2\text{CHCH}_2$, 1H); 4.98 (s, $\text{C}_6\text{H}_5\text{CH}_2\text{OCO}$, 2H); 5.23 (m, $\text{COOCH}_2\text{CHCH}_2$, 1H); 7.18 (m, $\text{NHCH}_2\text{CH}_2\text{NH}$, 2H); 7.33 (d, C_6H_5 , 2H); 7.38 (m, mPEG-OCONH, 1H); 7.52 (d, C_6H_5 , 2H).

25

30

The reaction scheme is illustrated in Fig. 6A.

Example 5

In vitro Cleavage of mPEG-DTB-DSPE Compound

5 *Ortho*-mPEG-DTB-DSPE and *para*- mPEG-DTB-DSPE (prepared as described in Example 1) were added to a buffered aqueous solution (pH 7.2) in the presence and absence of 150 μ M cysteine. Disappearance of the conjugates was monitored by HPLC (Phenomenex C₈ Prodigy, 4.6 x 50 mm column, detection at 277 nm, mobile phase methanol / water 95:5 with 0.1% trifluoroacetic acid at 1 mL/min). The results are
10 illustrated in Fig. 7A where the *ortho*-conjugate is represented by the open circles and the *para*-conjugate by the open squares.

Example 6

In vitro Cleavage of o- and p-mPEG-DTB-DSPE Compound in Liposomes

15 A. Liposome Preparation

The lipids partially hydrogenated phosphatidylcholine (PHPC), cholesterol and *ortho*- or *para*-mPEG-DTB-DSPE (prepared as described in Example 1, mPEG MW=1980 Daltons) were dissolved in a 95:5:3 molar ratio, respectively, in a suitable organic solvent, typically chloroform/methanol in a 1:1 or 1:3 ratio. The solvent was
20 removed by rotary evaporation to form a dried lipid film. The film was hydrated with aqueous buffer to form liposomes that were sized via extrusion to an average diameter of 120 nm.

B. *In vitro* Characterization

The liposomes were incubated in phosphate buffered saline, pH 7.2, containing 5
25 mM EDTA at 37°C in the presence of 150 μ M cysteine. Disappearance of the conjugates was monitored by HPLC (Phenomenex C₈ Prodigy, 4.6 x 50 mm column, detection at 277 nm, mobile phase methanol / water 95:5 with 0.1% trifluoroacetic acid at 1 mL/min). Results are shown in Fig. 7B where the liposomes comprising the *ortho*-conjugate are represented by the solid circles and liposomes comprising the *para*-
30 conjugate by the solid squares. The open circles and the open squares correspond to *ortho*-mPEG-DTB-DSPE and *para*-mPEG-DTB-DSPE in micellar form (discussed above in Example 5, Fig. 7A).

Example 7

In vitro Cleavage of *o*- and *p*-mPEG-DTB-DSPE Compound in Liposomes

A. Liposome Preparation

The lipids dioleoyl phosphatidylethanolamine (DOPE) and *ortho*- or *para*- mPEG-DTB-DSPE (prepared as described in Example 1, mPEG MW=1980 Daltons) were dissolved a 97:3 molar ratio in chloroform/methanol 1:1. The solvent was removed by rotary evaporation to form a dried lipid film. The lipid film was hydrated with an aqueous solution containing 30 mM each of the fluorophores *p*-xylene-bis-pyridinium bromide and trisodium 8-hydroxypyrenetrisulfonate. was hydrated with aqueous buffer to form liposomes that were sized via extrusion to an average diameter of 100 nm.

B. *In vitro* Characterization

The liposomes were incubated in HEPES buffer, pH 7.2, at 37°C in the presence of cysteine at concentrations of 15 μ M, 150 μ M, 300 μ M and 1.5 mM. Percent of released dye was determined as the increase in sample fluorescence ($\lambda_{em}=512$ nm, $\lambda_{ex}=413$ nm - pH-independent isobestic point) over that of the preincubation sample (zero release) normalized to the increase in fluorescence obtained after lysis of preincubation sample with 0.2% Triton X-100 (100% release) (Kirpotin, D. *et al.*, *FEBS Letters*, **388**:115-118 (1996)). Results at various cysteine concentrations for liposome comprising the *ortho*-compound are shown in Fig. 8A and for the *para*-compound are shown in Fig. 8B.

Example 8

In vivo Characterization of Liposomes comprising mPEG-DTB-DSPE Compound

A. Liposome Preparation

The lipids partially hydrogenated phosphatidylcholine (PHPC), cholesterol and *para*-mPEG-DTB-DSPE (prepared as described in Example 1, mPEG MW=1980 Daltons) were dissolved in a 55:40:5 mole percent ratio, respectively, in an organic solvent. The solvent was removed by rotary evaporation to form a dried lipid film. The film was hydrated with aqueous buffer containing diethylene triamine pentacetic acid (EDTA) to form liposomes. After downsizing the liposomes to an average

diameter of 120 nm unentrapped EDTA was removed and In^{111} was added to the external medium. The liposomes were incubated for a time sufficient for In^{111} to cross the lipid bilayer and chelate with EDTA.

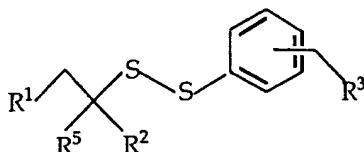
5 B. In vivo Administration

Mice were divided into two study groups. The liposome composition described above was injected into all test animals. One group of the test animals also received a 200 μL injection of 200 mM cysteine at 1, 3 and 5 hours post liposome injection. The other test group received an injection of saline at the same time points. Liposome
10 content in the blood was determined by monitoring blood samples for In^{111} . The results are shown in Fig. 10.

Although the invention has been described with respect to particular embodiments, it will be apparent to those skilled in the art that various changes and modifications can be made without departing from the invention.

We claim:

1. A compound having the general structure:



5

wherein R¹ is a hydrophilic polymer comprising a linkage for attachment to the dithiobenzyl moiety; R² is selected from the group consisting of H, alkyl and aryl; R³ is selected from the group consisting of O(C=O)R⁴, S(C=O)R⁴, and O(C=S)R⁴; R⁴ comprises an amine-containing ligand; and R⁵ is selected from the group consisting of H, alkyl and aryl; and where orientation of CH₂-R³ is selected from the ortho position and the para position.

2. The compound of claim 1, wherein R⁵ is H and R² is selected from the group consisting of CH₃, C₂H₅ and C₃H₈.

15

3. The compound according to claim 1 or claim 2, wherein the amine-containing ligand R⁴ is selected from the group consisting of a polypeptide, an amine-containing drug and an amine-containing lipid.

20

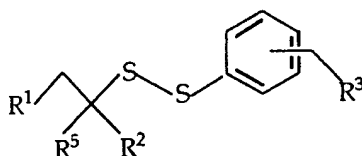
4. The compound according to claim 1 or claim 2, wherein the amine-containing ligand R⁴ is an amine-containing lipid comprising either a single hydrocarbon tail or a double hydrocarbon tail.

5. The compound of claim 4, wherein the amine-containing lipid is a phospholipid having a double hydrocarbon tail.

25

6. The compound according to any one of claims 1, 3, 4 or 5, wherein R² and R⁵ are alkyls.

7. The compound according to any of the preceding claims, wherein R¹ is selected from the group consisting of polyvinylpyrrolidone, polyvinylmethylether, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyloxazoline, polyhydroxypropyl-methacrylamide, polymethacrylamide, polydimethyl-acrylamide, polyhydroxypropylmethacrylate, polyhydroxyethylacrylate, hydroxymethylcellulose, hydroxyethylcellulose, polyethyleneglycol, polyaspartamide, copolymers thereof, and polyethyleneoxide-polypropylene oxide.
8. The compound according to any one of claims 1-6, wherein R¹ is polyethyleneglycol.
9. The compound of claim 8, wherein R⁵ is H and R² is CH₃ or C₂H₅.
10. A liposome comprising the compound of any of the preceding claims.
11. The compound according to any one of claims 1, 2, 7, 8 or 9, wherein the amine-containing ligand R⁴ is a polypeptide.
12. The compound of claim 11, wherein the polypeptide is a recombinant polypeptide.
13. The compound of claim 11, wherein the polypeptide is a cytokine.
14. The compound of claim 11, wherein the polypeptide is selected from the group consisting of interferons, interleukins, growth factors, and enzymes.
15. A composition comprising a conjugate obtainable by reaction with a compound having the general structural formula:



wherein R^1 is a hydrophilic polymer comprising a linkage for attachment to the dithiobenzyl moiety; R^2 is selected from the group consisting of H, alkyl and aryl; R^3 is
 5 selected from the group consisting of $O(C=O)R^4$, $S(C=O)R^4$, and $O(C=S)R^4$; R^4 comprises a leaving group; and R^5 is selected from the group consisting of H, alkyl and aryl; and where orientation of CH_2-R^3 is selected from the ortho position and the para position; and

a pharmaceutically-acceptable carrier.

10

16. The composition of claim 15, wherein R^2 is selected from the group consisting of CH_3 , C_2H_5 and C_3H_8 .

15

17. The composition of claim 15, wherein R^3 is $O(C=O)R^4$ and R^4 is a hydroxy- or oxy-containing leaving group.

20

18. The composition of claim 15, wherein the leaving group is derived from a compound selected from the group consisting of chloride, *para*-nitrophenol, *ortho*-nitrophenol, N-hydroxy-tetrahydrophthalimide, N-hydroxysuccinimide, N-hydroxy-glutarimide, N-hydroxynorbornene-2,3-dicarboxyimide, 1-hydroxybenzotriazole, 3-hydroxypyridine, 4-hydroxypyridine, 2-hydroxypyridine, 1-hydroxy-6-trifluoromethylbenzotriazole, imidazole, triazole, N-methyl-imidazole, pentafluorophenol, trifluorophenol and trichlorophenol.

25

19. The composition of claim 15, wherein said compound is reacted with an amine-containing ligand that displaces R^4 to form a conjugate comprising said amine-containing ligand.

20. The composition of claim 19, wherein the amine-containing ligand comprises a phospholipid.

21. The composition of claim 19, wherein the amine-containing ligand comprises
5 a polypeptide.

22. The composition according to claim 20 or claim 21, wherein R¹ is selected from the group consisting of polyvinylpyrrolidone, polyvinylmethylether, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyloxazoline, polyhydroxypropyl-methacrylamide, polymethacrylamide, polydimethylacrylamide,
10 polyhydroxypropylmethacrylate, polyhydroxyethylacrylate, hydroxymethylcellulose, hydroxyethylcellulose, polyethyleneglycol, polyaspartamide, copolymers thereof, and polyethyleneoxide-polypropylene oxide.

23. The composition according to claim 20 or claim 21, wherein R¹ comprises
15 polyethyleneglycol.

24. The composition of claim 23, wherein R² is CH₃ or C₂H₅.

25. The composition of claim 20, wherein the composition containing the
20 conjugate comprises a liposome.

26. The composition of claim 25, wherein the liposome further comprises an entrapped therapeutic agent.

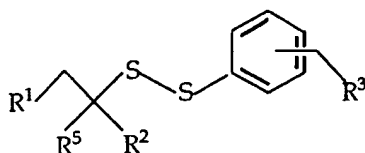
27. The composition of claim 21, wherein the polypeptide comprises a
25 recombinant polypeptide.

28. The composition of claim 21, wherein the polypeptide comprises a cytokine.

29. The composition of claim 21, wherein the polypeptide is selected from the group consisting of interferons, interleukins, growth factors, and enzymes.

30. A liposome composition comprising:

5 liposomes comprising a surface coating of hydrophilic polymer chains wherein at least a portion of the hydrophilic polymer chains have the general structure:



wherein R¹ is a hydrophilic polymer comprising a linkage for attachment to the
 10 dithiobenzyl moiety; R² is selected from the group consisting of H, alkyl and aryl; R³ is selected from the group consisting of O(C=O)R⁴, S(C=O)R⁴, and O(C=S)R⁴; R⁴ comprises an amine-containing ligand; and R⁵ is selected from the group consisting of H, alkyl and aryl; and where orientation of CH₂-R³ is selected from the ortho position and the para position,

15 wherein the liposomes have a longer blood circulation lifetime than liposomes having hydrophilic polymer chains joined to the liposome via an aliphatic disulfide linkage.

31. The composition of claim 30, wherein R⁵ is H and R² is selected from the group consisting of CH₃, C₂H₅ and C₃H₇.

20

32. The composition according to claim 30 or claim 31, wherein the amine-containing lipid comprises a phospholipid.

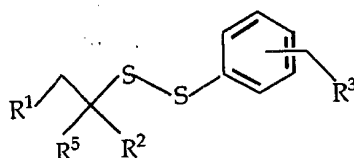
33. The composition according to any one of claims 30-32, wherein R¹ is
 25 selected from the group consisting of polyvinylpyrrolidone, polyvinylmethylether, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyloxazoline, polyhydroxypropyl-methacrylamide, polymethacrylamide, polydimethyl-acrylamide, polyhydroxypropylmethacrylate, polyhydroxyethylacrylate, hydroxymethylcellulose,

hydroxyethylcellulose, polyethyleneglycol, polyaspartamide, copolymers thereof, and polyethyleneoxide-polypropylene oxide.

34. The composition according to any one of claims 30-32, wherein R¹ comprises
5 polyethyleneglycol.

35. The composition according to any one of claims 30-34, wherein the liposome further comprises an entrapped therapeutic agent.

10 36. A method for improving the blood circulation lifetime of liposomes having a surface coating of releasable hydrophilic polymer chains, comprising
preparing liposomes that include between about 1% to about 20% of a compound having the general structure:



15

wherein R¹ is a hydrophilic polymer comprising a linkage for attachment to the dithiobenzyl moiety; R² is selected from the group consisting of H, alkyl and aryl; R³ is selected from the group consisting of O(C=O)R⁴, S(C=O)R⁴, and O(C=S)R⁴; R⁴
20 comprises an amine-containing lipid; and R⁵ is selected from the group consisting of H, alkyl and aryl; and where orientation of CH₂-R³ is selected from the ortho position and the para position.

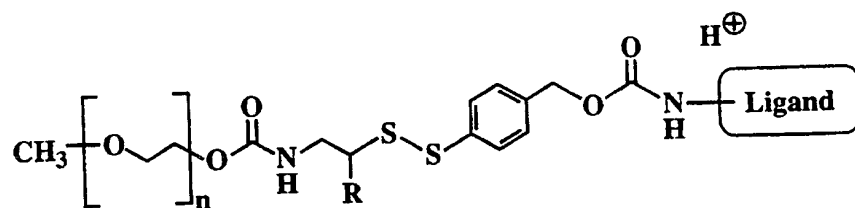
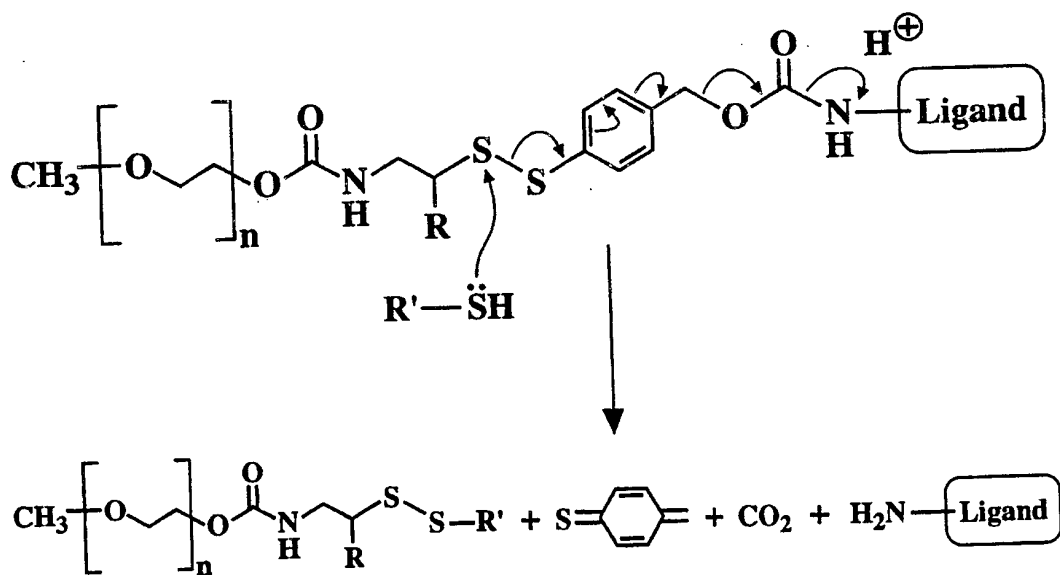
37. The method of claim 36, wherein R⁵ is H and R² is selected from the group
25 consisting of CH₃, C₂H₅ and C₃H₈.

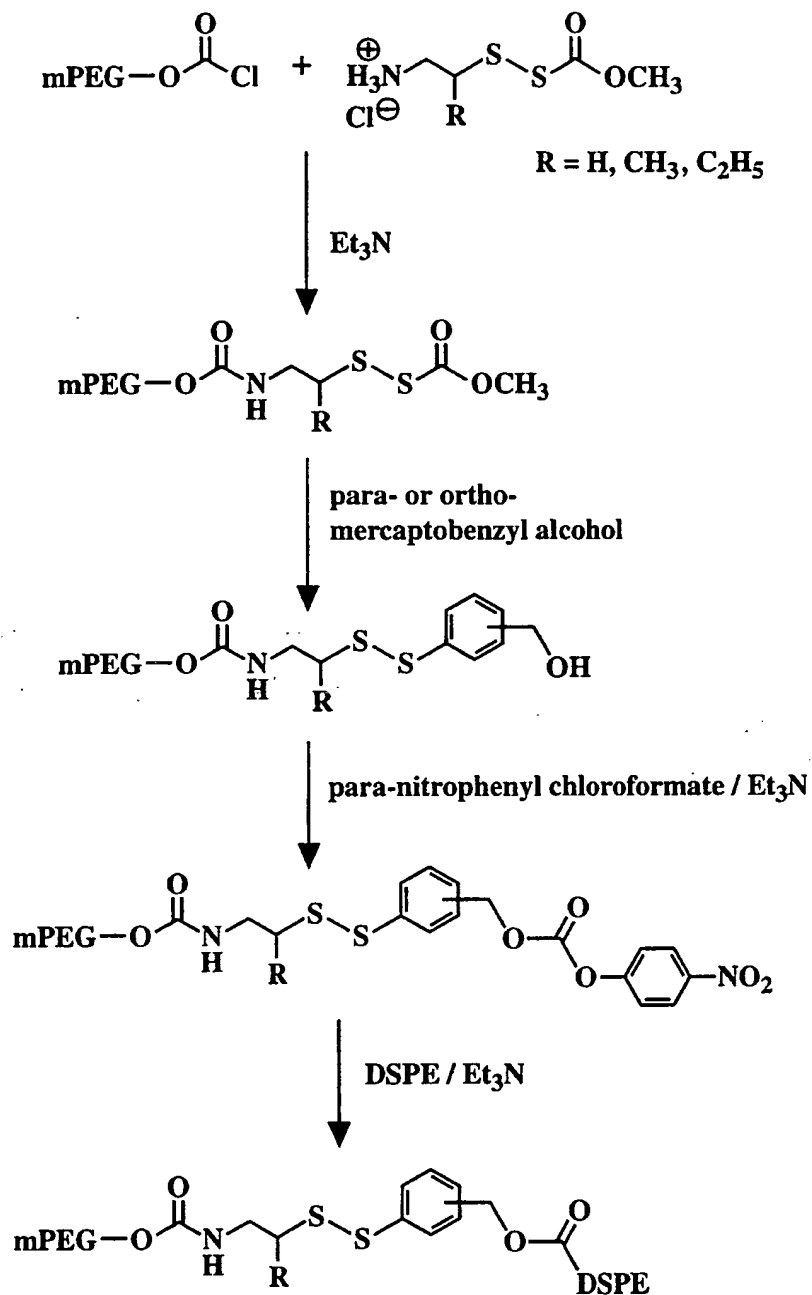
38. The method according to claim 36 or claim 37, wherein the amine-containing lipid comprises a phospholipid.

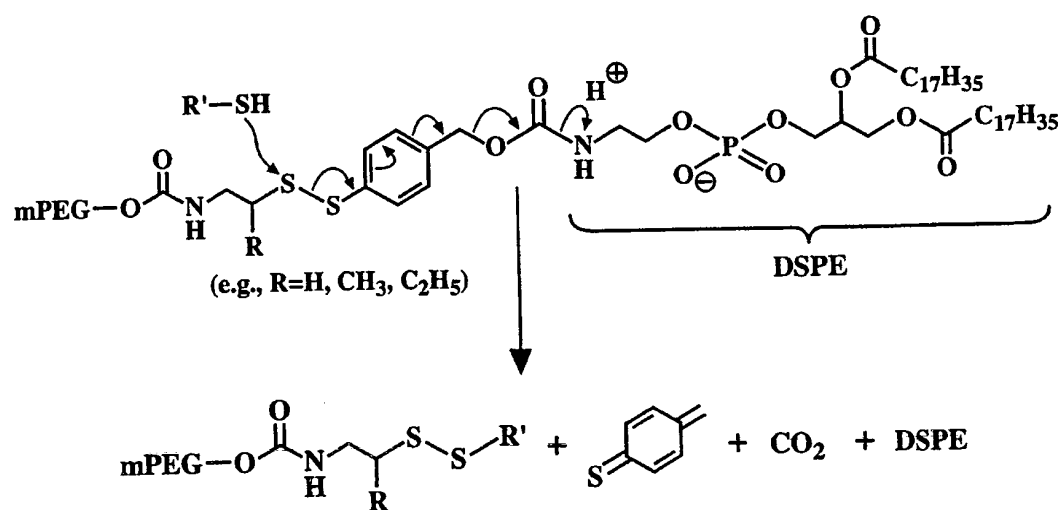
39. The method according to any one of claims 36-38, wherein R¹ is selected from the group consisting of polyvinylpyrrolidone, polyvinylmethylether, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyloxazoline, polyhydroxypropyl-methacrylamide, polymethacrylamide, polydimethyl-acrylamide, polyhydroxypropylmethacrylate, polyhydroxyethylacrylate, hydroxymethylcellulose, hydroxyethylcellulose, polyethyleneglycol, polyaspartamide, copolymers thereof, and polyethyleneoxide-polypropylene oxide.

40. The method according to any one of claims 36-38, wherein R¹ comprises polyethyleneglycol.

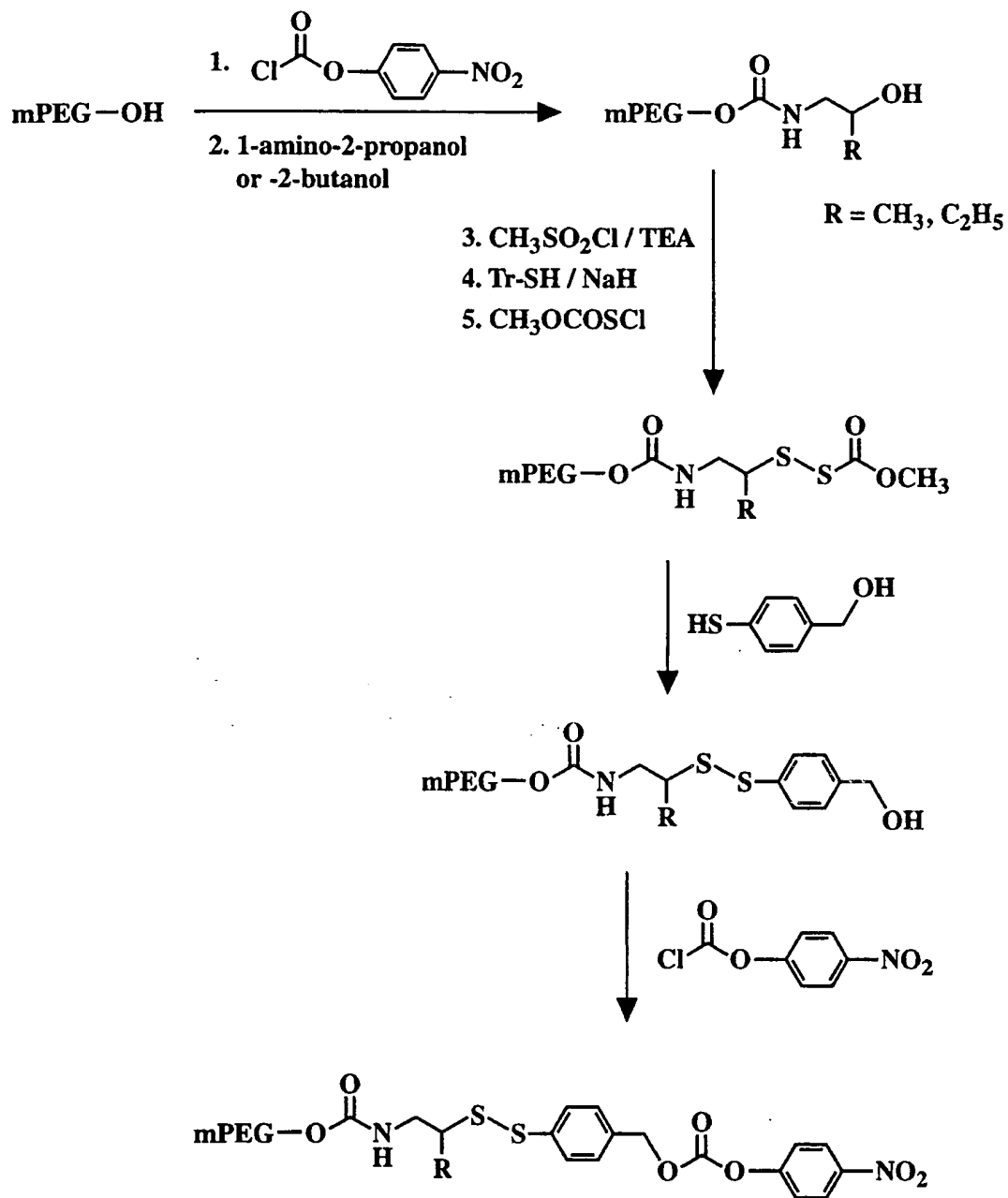
41. The method according to any one of claims 36-40, wherein the liposome further comprises an entrapped therapeutic agent.

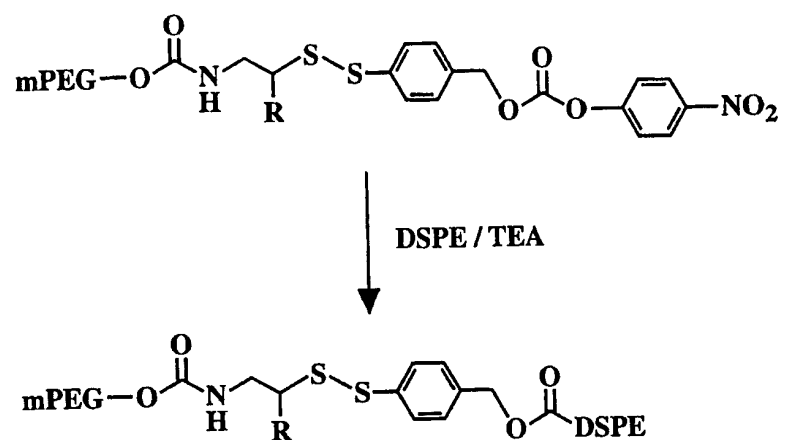
**Fig. 1A****Fig. 1B**

**Fig. 2**

**Fig. 3**

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**Fig. 4A**

**Fig. 4B**

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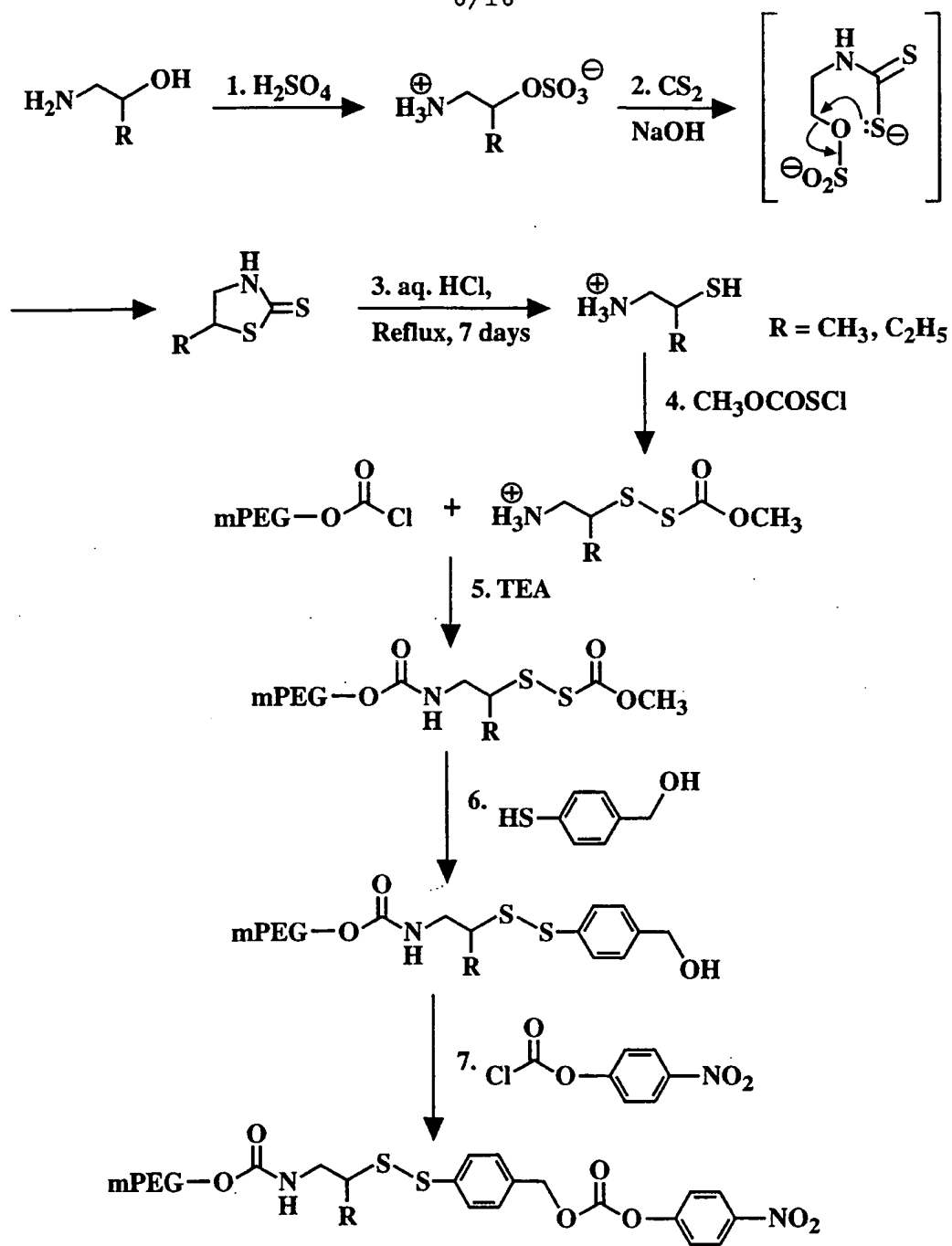
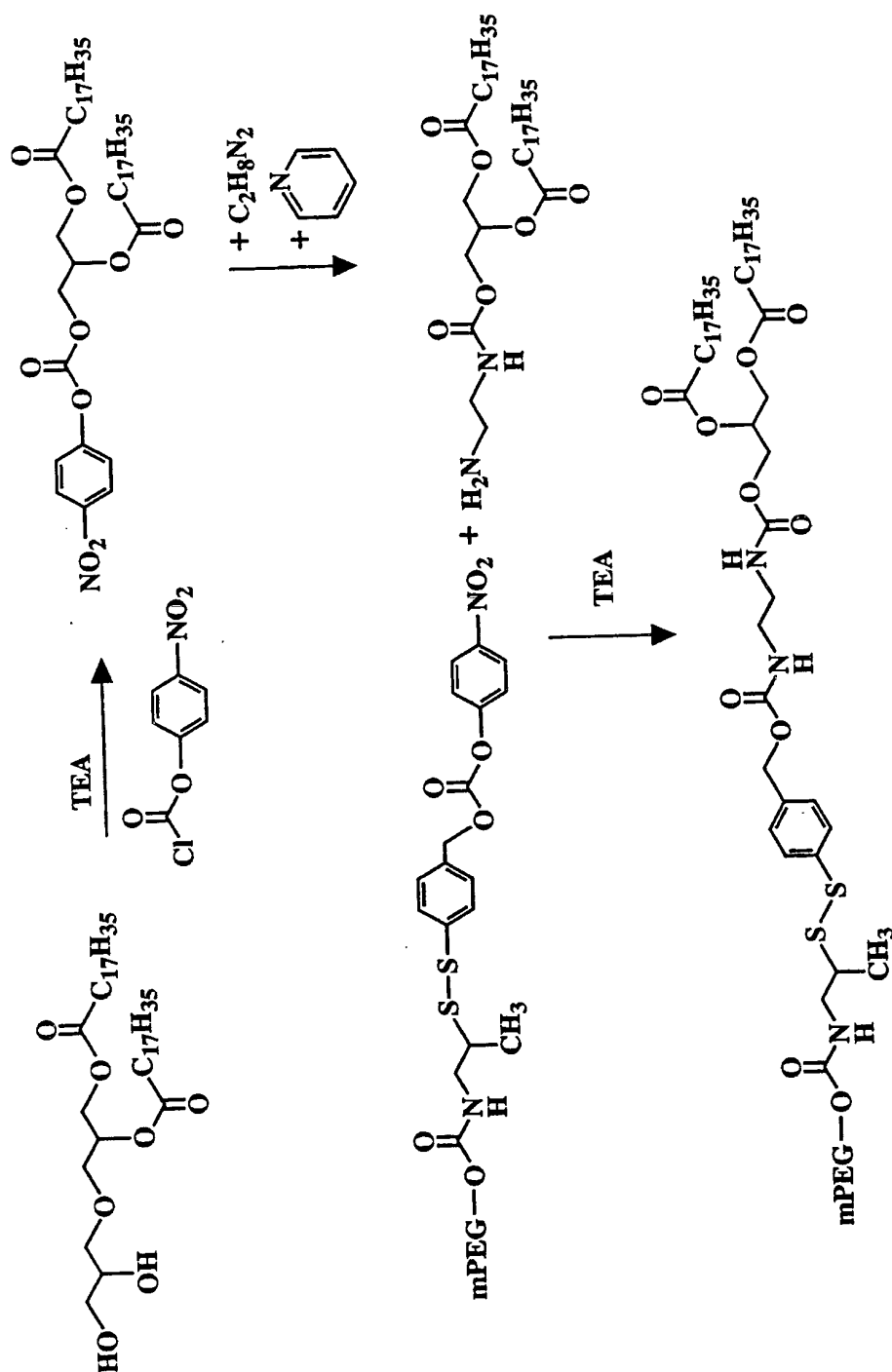
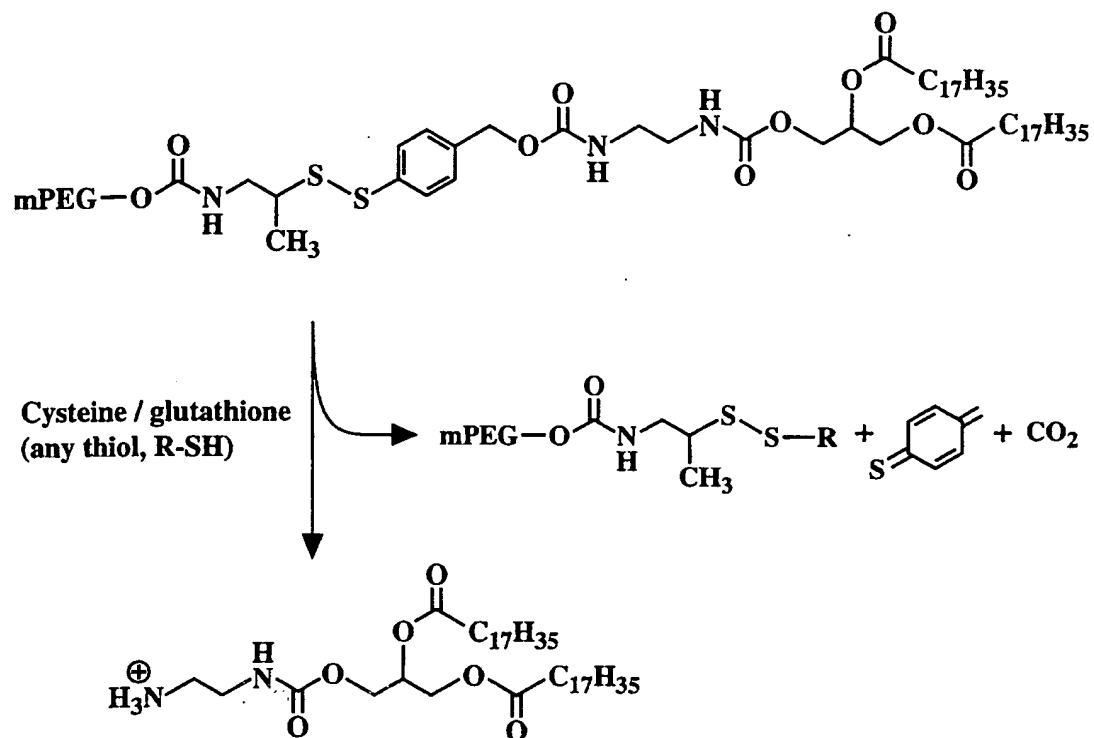


Fig. 5

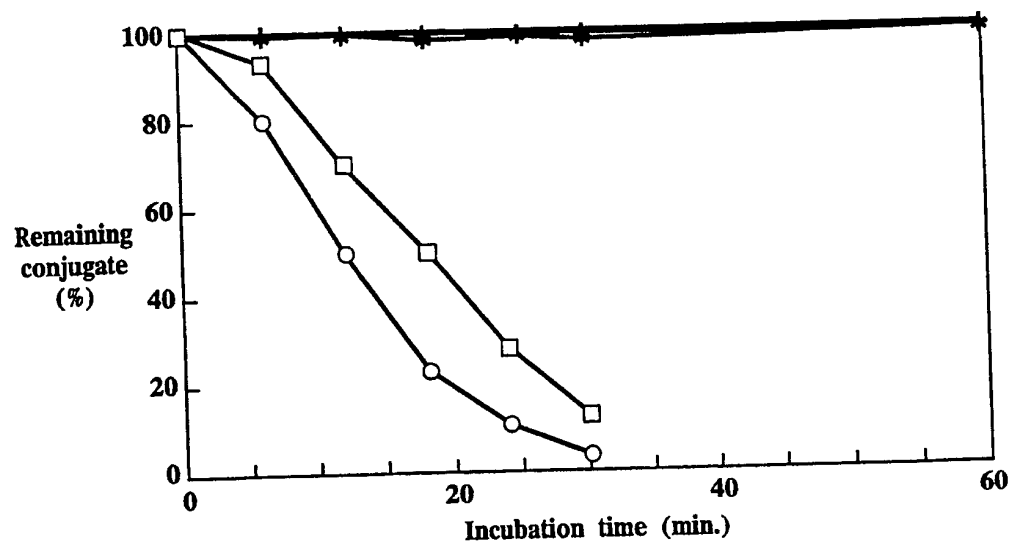
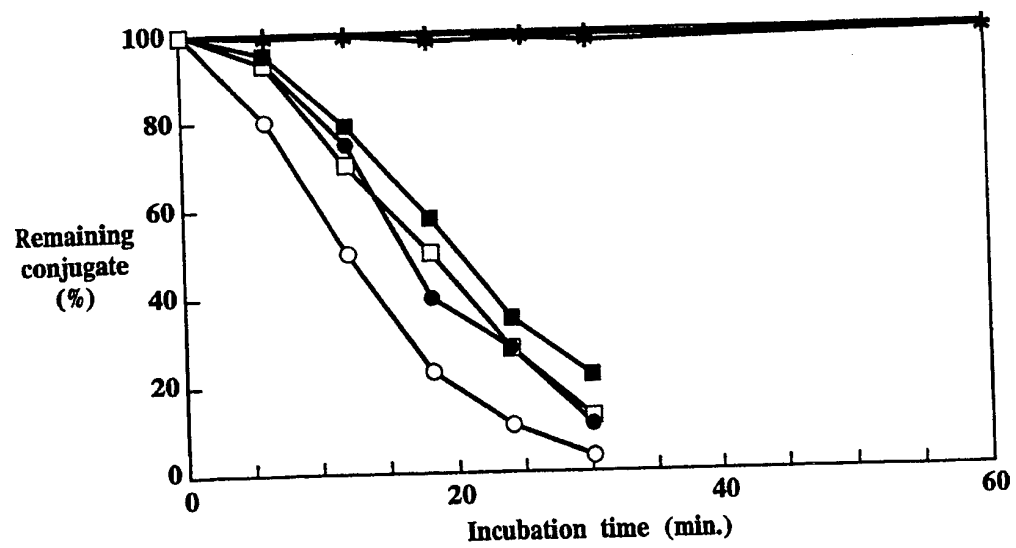
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**Fig. 6A**

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**Fig. 6B**

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**Fig. 7A****Fig. 7B**

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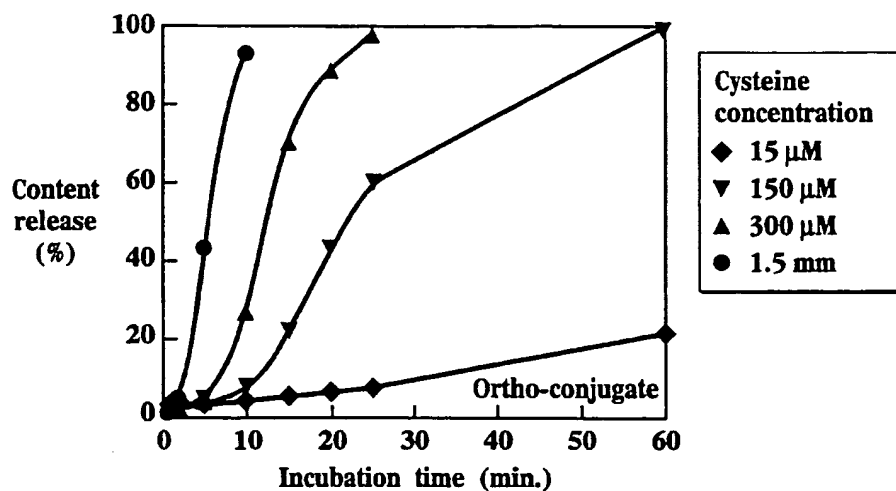


Fig. 8A

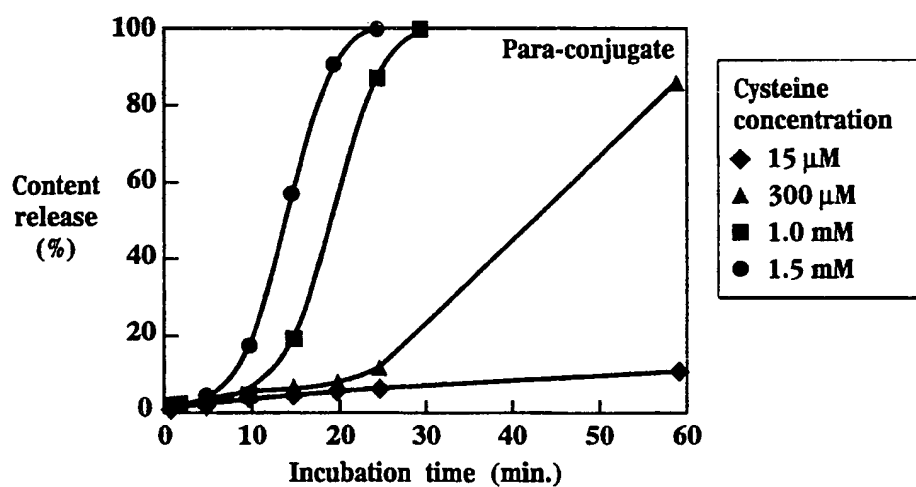


Fig. 8B

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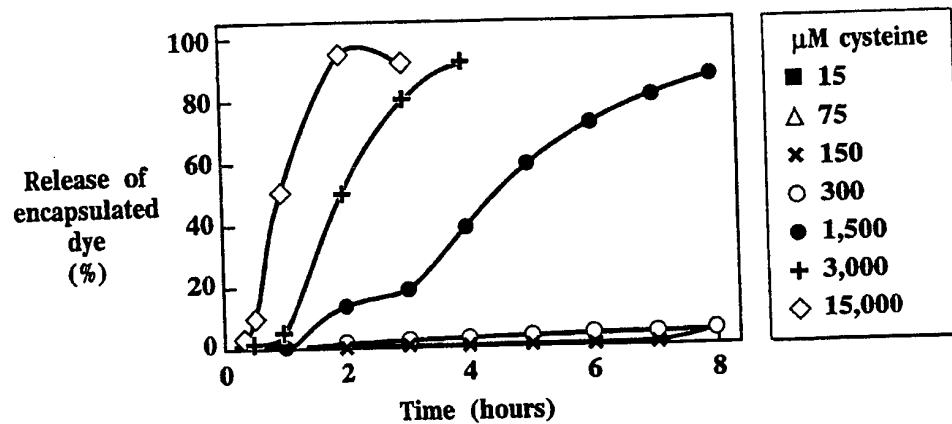


Fig. 9A

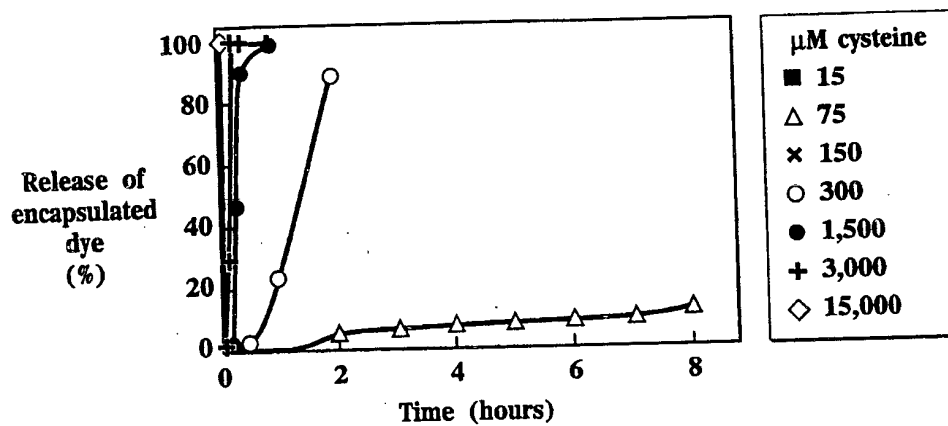


Fig. 9B

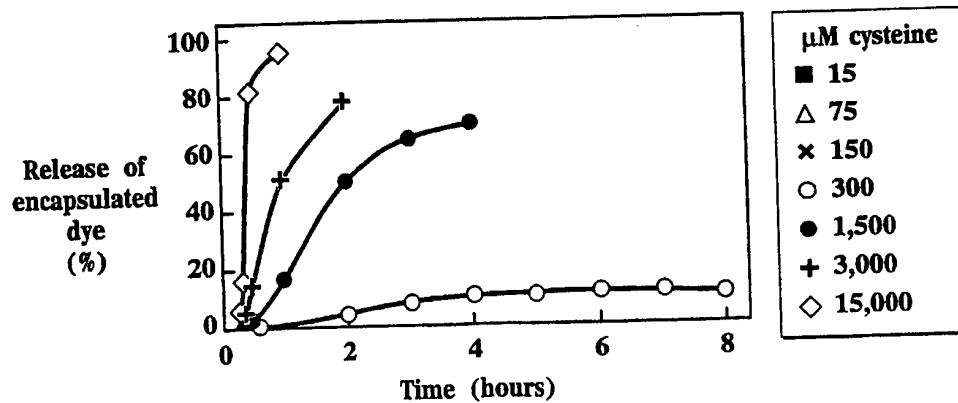
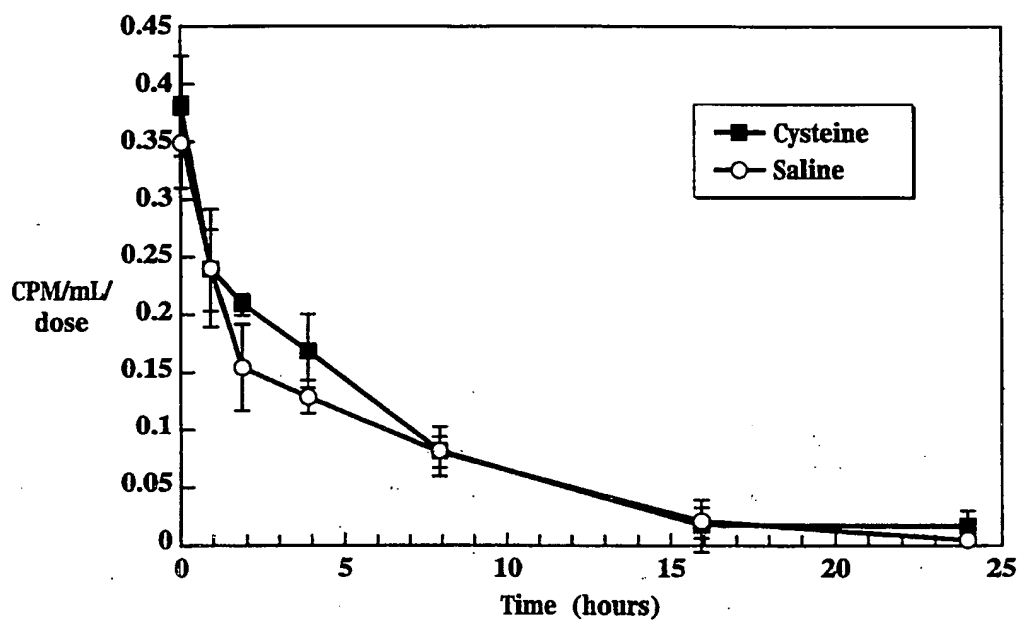
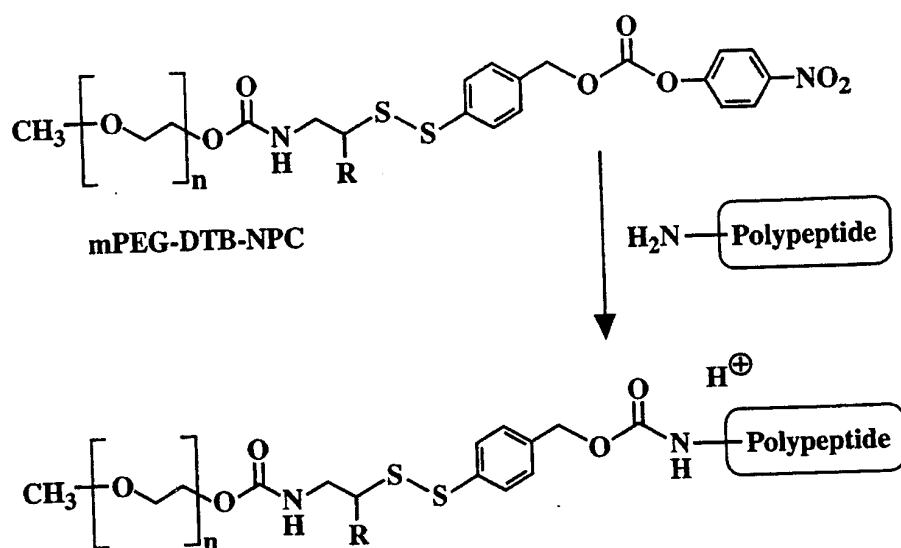
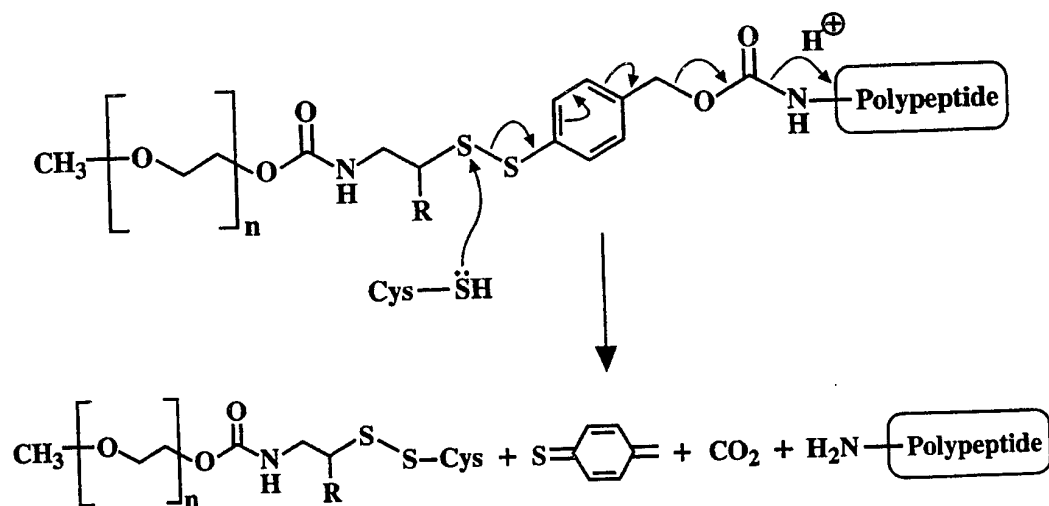


Fig. 9C

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**Fig. 10**

**Fig. 11A****Fig. 11B**

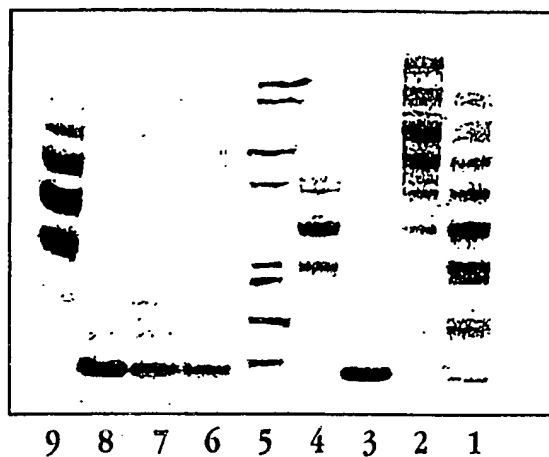
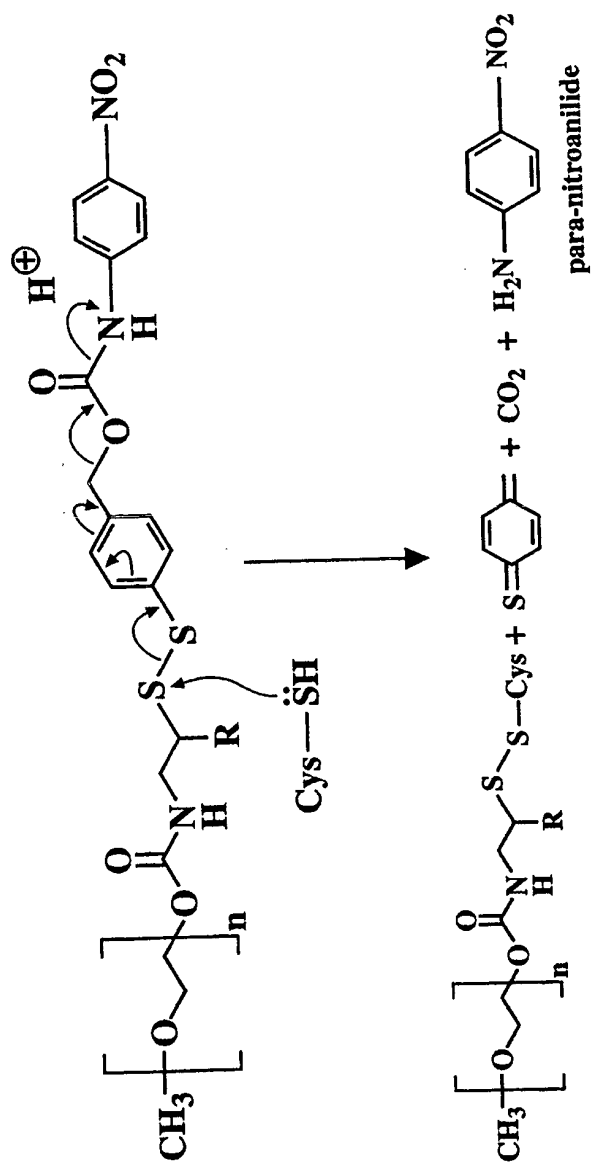
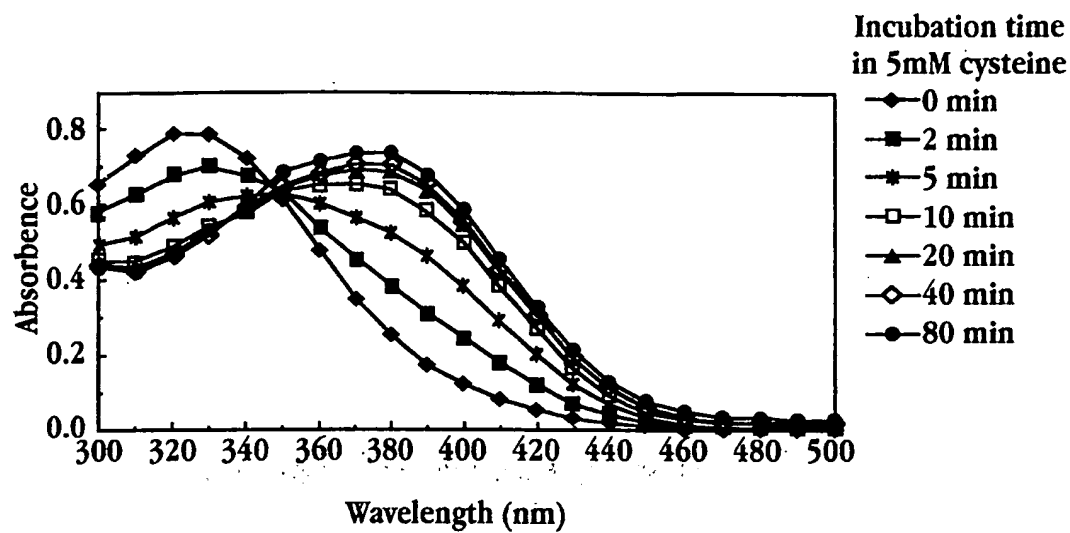
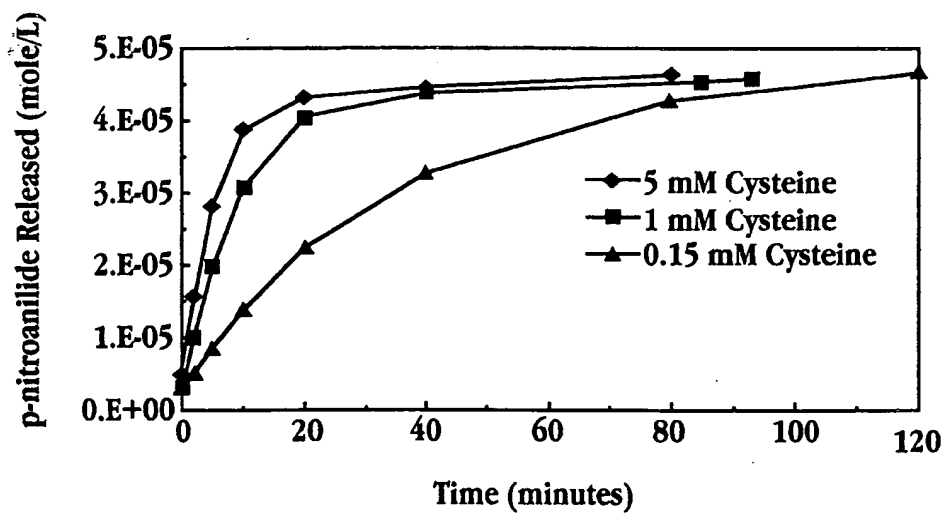


Fig. 12

**Fig. 13**

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**Fig. 14A****Fig. 14A**

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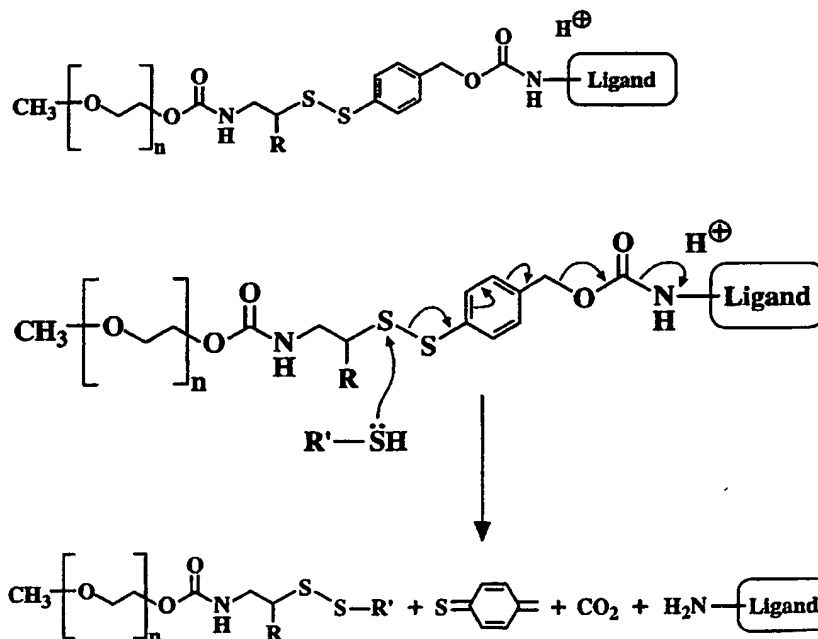
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60/130,897 23 April 1999 (23.04.1999) US
- (71) Applicant: ALZA CORPORATION [US/US]; 1900 Charleston Road, P.O. Box 7210, Mountain View, CA 94039-7210 (US).
- (72) Inventor: ZALIPSKY, Samuel; 1202 Truman Street, Redwood City, CA 94061 (US).
- (74) Agents: SIMBOLI, Paul, B. et al.; ALZA Corporation, 1900 Charleston Road, P.O. Box 7210, Mountain View, CA 94039-7210 (US).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
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(54) Title: RELEASABLE LINKAGE AND COMPOSITIONS CONTAINING SAME



(57) Abstract: A compound comprised of a hydrophilic polymer covalently yet reversibly linked to an amine-containing ligand through a dithiobenzyl linkage is described.

INTERNATIONAL SEARCH REPORT

Int lional Application No

PCT/US 00/10830

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K47/48 A61K9/127 C07F9/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C07F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

PAJ, WPI Data, EPO-Internal, BIOSIS, BEILSTEIN Data, CHEM ABS Data, MEDLINE, CANCERLIT, EMBASE, DISSERTATION ABS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIRPOTIN DMITRI ET AL: "Liposomes with detachable polymer coating: Destabilization and fusion of dioleoylphosphatidylethanolamine vesicles triggered by cleavage of surface-grafted poly(ethylene glycol)." FEBS LETTERS, vol. 388, no. 2-3, 1996, pages 115-118, XP002080855 ISSN: 0014-5793 abstract page 115, paragraph INTRODUCTION figures 1,3 page 117, left-hand column, line 1 -right-hand column, line 5 --- -/--	1-41

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/10830

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 891 468 A (ZALIPSKY SAMUEL ET AL) 6 April 1999 (1999-04-06) example 5 figure 11 ---	1-41
X	JOHNSSON MARKUS ET AL: "Optimization of drug loading procedures and characterization of liposomal formulations of two novel agents intended for boron neutron capture therapy (BNCT)." JOURNAL OF LIPOSOME RESEARCH, vol. 9, no. 1, February 1999 (1999-02), pages 53-79, XP000805700 ISSN: 0898-2104 abstract page 57 ---	1-41
P,X	ZALIPSKY SAMUEL ET AL: "New detachable poly(ethylene glycol) conjugates: Cysteine-cleavable lipopolymers regenerating natural phospholipid, diacyl phosphatidylethanolamine." BIOCONJUGATE CHEMISTRY, vol. 10, no. 5, 20 September 1999 (1999-09-20), pages 703-707, XP002159601 ISSN: 1043-1802 the whole document ---	1-41
Y	MUELLER C E ET AL: "AMPHIPHILIC UNSYMMETRICAL DISULFIDES AS BILAYER-FORMING COMPOUNDS" ARCHIV DER PHARMAZIE (WEINHEIM), vol. 322, no. 6, 1989, pages 343-350, XP000981914 ISSN: 0365-6233 figures page 346, left-hand column, line 4 -right-hand column, line 7 ---	1-41
Y	GABER MOHAMED H ET AL: "Thermosensitive sterically stabilized liposomes: Formulation and in vitro studies on mechanism of doxorubicin release by bovine serum and human plasma." PHARMACEUTICAL RESEARCH (NEW YORK), vol. 12, no. 10, 1995, pages 1407-1416, XP000979539 ISSN: 0724-8741 table 1 page 1414, paragraph DISCUSSION -page 1415, right-hand column, last paragraph --- -/--	1-41

INTERNATIONAL SEARCH REPORT

Int lional Application No
PCT/US 00/10830

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>VAAGE JAN ET AL: "Therapy of human ovarian carcinoma xenografts using doxorubicin encapsulated in sterically stabilized liposomes." CANCER (PHILADELPHIA), vol. 72, no. 12, 1993, pages 3671-3675, XP000979540 ISSN: 0008-543X page 3672, left-hand column, last paragraph -right-hand column, line 11 tables 1-3 page 3674, paragraph DISCUSSION</p>	1-41
Y	<p>VAAGE JAN ET AL: "Therapy of a xenografted human colonic carcinoma using cisplatin or doxorubicin encapsulated in long-circulating pegylated stealth liposomes." INTERNATIONAL JOURNAL OF CANCER, vol. 80, no. 1, 5 January 1999 (1999-01-05), pages 134-137, XP000979545 ISSN: 0020-7136 abstract page 134, right-hand column, line 15 - line 22 figures 1,2 tables 1,2</p>	1-41
Y	<p>VAAGE JAN ET AL: "Tissue distribution and therapeutic effect of intravenous free or encapsulated liposomal doxorubicin on human prostate carcinoma xenografts." CANCER (PHILADELPHIA), vol. 73, no. 5, 1994, pages 1478-1484, XP000979547 ISSN: 0008-543X abstract page 1479, left-hand column, last paragraph -right-hand column, last paragraph page 1483, paragraph DISCUSSION</p>	1-41
Y	<p>VAAGE J ET AL: "THERAPY OF PRIMARY AND METASTATIC MOUSE MAMMARY CARCINOMAS WITH DOXORUBICIN ENCAPSULATED IN LONG CIRCULATING LIPOSOMES" INTERNATIONAL JOURNAL OF CANCER, vol. 51, no. 6, 1992, pages 942-948, XP000979548 ISSN: 0020-7136 page 943, left-hand column, last paragraph tables figures</p>	1-41

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INTERNATIONAL SEARCH REPORT

Int. :ional Application No

PCT/US 00/10830

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>HIROTA SADA0: "Physicochemical specification of drug carrying liposomes for the quality control in the industrial production." INTERNATIONAL JOURNAL OF PHARMACEUTICS (AMSTERDAM), vol. 162, no. 1-2, 20 March 1998 (1998-03-20), pages 185-194, XP000979551 ISSN: 0378-5173 tables 7-10</p> <p>-----</p>	1-41

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-41 in part

Present claims 1-14 relate to an extremely large number of possible compounds. Present claims 15-35 relate to an extremely large number of possible compositions containing these compounds. Present claims 36-41 relate to an extremely large number of possible methods using these compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only one single of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compound as mentioned in the examples, and to compositions and methods in which this compound is used.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/10830

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5891468 A	06-04-1999	US 6056973 A	02-05-2000
		AU 715063 B	13-01-2000
		AU 4987897 A	11-05-1998
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